424 Rec'd PCT/PTO 21 APR 2000

Form PTO-1390 (REV 10-94) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 641050.90013

U.S APPLICATION NO (If known, see 37 C.F.R 1.5): 09/529925

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA98/00992	26 October 1998	24 October 1997

TITLE OF INVENTION: P-40/ANNEXIN I AND RELATED PROTEINS AND THEIR ROLE IN MULTIDRUG RESISTANCE

APPLICANT(S) FOR DO/EO/US: GEORGES, Elias; WANG, Ying

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau)
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau)
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 in included.
13. [XX] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Postcard Receipt

EXPRESS MAIL NO. EL389230589US

(January 1997)

QB

U S APPLICATION NO (If known, see 37 CFR 1.5) 09/529925	INTERNATIONAL APPLICATION NO PCT/CA98/00992	ATTORNEY'S DOCKET NUMBER 641050.90013

17. [ ] The following fees are submitted:	CALCULATIONS ONLY	PTO USE
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO..... $840.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... $670.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))..... $690.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... $ 970.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... $96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 -20 =		X $18.00	$	
Independent claims	5 -3 =	2	X $78.00	$156.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)	6		X $260.00	$996.00	
TOTAL OF ABOVE CALCULATIONS =				$996.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$996.00	
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.429(f)). +				$	
TOTAL NATIONAL FEE =				$996.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$996.00	
				Amount to be: refunded	$
				charged	$

a. [ ] A check in the amount of $_____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 17-0055 in the amount of $996.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No 17-0055. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

SIGNATURE Jean C. Baker

NAME Jean C. Baker

REGISTRATION NUMBER Reg. No. 35,433

(1)

QB

#3

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27 (d)) - NONPROFIT ORGANIZATION	Docket No. 641050.90013

Serial No.	Filing Date	Patent No	Issue Date
Unknown	Unknown		

Applicant/
Patentee GEORGES et al.

Invention.

P-40/ANNEXIN I AND RELATED PROTEINS AND THEIR ROLE IN MULTIDRUG RESISTANCE

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION. McGILL UNIVERSITY
ADDRESS OF ORGANIZATION 845 Sherbrooke Street West
Montreal, Quebec, Canada, H3A 1B1

TYPE OF NONPROFIT ORGANIZATION:

- ☒ University or other Institute of Higher Education
- ☐ Tax Exempt under Internal Revenue Service Code (26 U.S.C 501(a) and 501(c)(3))
- ☐ Nonprofit Scientific or Educational under Statute of State of The United States of America
  Name of State Citation of Statute
- ☐ Would Qualify as Tax Exempt under Internal Revenue Service Code (26 U.S.C 501(a) and 501(c)(3)) if Located in The United States of America
- ☐ Would Qualify as Nonprofit Scientific or Educational under Statute of State of The United States of America if Located in The United States of America
  Name of State Citation of Statute

I hereby declare that the above-identified nonprofit organization qualifies as a nonprofit organization as defined in 37 C.F.R. 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in

- ☐ the specification to be filed herewith.
- ☒ the application identified above
- ☐ the patent identified above

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention

If the rights held by the above-identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e)

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below

☒ no such person, concern or organization exists
☐ each such person, concern or organization is listed below.

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☒ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed

NAME OF PERSON SIGNING: Dr. Alex NAVARRE

TITLE IN ORGANIZATION: Director, Office of Technology Transfer

ADDRESS OF PERSON SIGNING: 3550 University Street
Montreal, Quebec, Canada, H3A 2A7

SIGNATURE: _____ DATE: 16/06/2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	**GEORGES et al.**	Docket No.:	641050.90013
Serial No.:	**Unassigned**	Filed:	**Concurrently herewith**
Int'l appln No.:	**PCT/CA98/00992**	Int'l filing date:	**26 October 1998**
Title:	**P-40/ANNEXIN I AND RELATED PROTEINS AND THEIR ROLE IN MULTIDRUG RESISTANCE**		

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PRELIMINARY AMENDMENT

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In connection with the above-identified application filed herewith, please enter the following preliminary amendment:

IN THE CLAIMS:

Please cancel claims 1-5; 8-14; and 28-29 without prejudice.

Please amend the claims as follows:

15. (Amended) A method of identifying a compound[s] that affects Annexin-based MDR in a cell, said method comprising:

a) incubating said cell in the presence of a potential Annexin-based MDR-affecting compound in the presence and absence of a drug; and

b) assessing the effect of said compound on the resistance of said cell to said drug.

16. (Amended) The method of claim [14] 15, wherein said cell is a cell [in accordance with one of claims 13 or 14] having been rendered multidrug resistant (MDR) by an expression of an Annexin nucleic acid molecule.

17. (Amended) The method of claim 15 [or 16], wherein said compound is selected from the group consisting of a nucleic acid molecule encoding an Annexin variant, or a part thereof, a dominant negative mutant of an Annexin, a mutant Annexin, an antibody to Annexin, a peptide, and a small molecule.

19. (Amended) The method of [one of] claim[s] 15[, 16, 17 or 18], wherein said drug is an anticancer drug.

20. (Amended) A method of reducing Annexin-based MDR in a cell comprising: administering thereto [a therapeutically] an effective amount of a compound selected from the group consisting of a nucleic acid molecule, a dominant negative mutant of an Annexin, a mutant Annexin protein, an antibody to Annexin, a peptide, and a small molecule, whereby said effective amount of said compound reduces Annexin-based MDR in said cell.

26. (Amended) A method of diagnosing the presence or predisposition of Annexin-based MDR in a [patient] sample comprising:

[a) taking a sample from said patient;]

[b)] a) determining the amount of Annexin protein and/or nucleic acid in said sample; and

[c)] b) diagnosing the presence or predisposition of Annexin-based MDR in said [patient] sample, wherein an increased amount of said Annexin protein and/or nucleic acid in said sample as compared to a control sample indicates the presence or predisposition towards Annexin-based MDR.

Please add the following new claims:

--30. The method of claim 27, wherein said sample is from a patient.

31. The method of claim 27, wherein said sample is a sample from a pathogen.--

Affirmation is hereby given by the foregoing, that claims 1-5; 8-14; and 28-29 have been withdrawn from further consideration. Applicants reserve the right to prosecute the subject-matter thereof by way of a divisional.

Applicant respectfully requests that the preliminary amendment described herein be entered into the record prior to examination and consideration of the above-identified application.

Respectfully submitted,

QUARLES & BRADY

by: Jean C. Baker

Jean C. Baker
Registration No. 35,433

Date: April 20, 2000

QUARLES & BRADY
411 East Wisconsin Avenue
Milwaukee WI 53202-4497
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TITLE OF THE INVENTION

P-40/ANNEXIN I AND RELATED PROTEINS AND THEIR ROLE I MULTIDRUG RESISTANCE

FIELD OF THE INVENTION

The present invention relates in general to multidrug resistance (MDR) in cells. In particular, the present invention relates to the identification of a new member of the MDR gene family, P-40, as well as to the identification of P-40 related genes (homologs) as being further members of the MDR gene family. The present invention therefore relates to nucleic acid molecules encoding P-40 protein and P-40 proteins homologs, to multidrug resistant cells containing these nucleic acid molecules; to hybridomas containing antibodies to P-40 and P-40 homologs; to nucleic acid probes for the detection of these nucleic acid molecules; to a method of detection of such nucleic acid molecules or of the P-40 protein or P-40 homologs; to bioassays comprising the nucleic acid molecules encoding P-40 or P-40 homologs, P-40 protein or P-40 protein homologs, or antibodies of the present invention to diagnose, assess or prognose MDR in an animal; to therapeutic uses of the nucleic acid molecules of the present invention (i.e. antisense), protein or antibodies of the present invention; and to methods of preventing MDR in an animal.

BACKGROUND OF THE INVENTION

The ability of malignant cells to develop resistance to multiple anticancer drugs is a major obstacle in the treatment of cancers (Ferguson et al., 1989, Cancer Bulletin 41(1): 7-13). Studies using *in vitro* model systems have led to the identification of several proteins which confer resistance to different classes of anticancer drugs (Pastan et al., 1987, New England J. Med. 316(22): 1388-1393;Bradley et al., 1988, Biochim. Biophys. Acta 948: 87-128). The overexpression of P-glycoprotein (P-gp) and the multidrug

resistance associated protein (MRP) in cells selected with hydrophobic cytotoxic drugs (*Vinca alkaloids*, anthracyclinses and epipodophyllotoxins) have been shown to confer a multidrug resistance phenotype (Gottesman et al., 1993, Ann. Rev. Biochem. 62: 385-427; Endicott et al., 1989, Ann. Rev. of Biochem. 58: 137-171; Cole et al., 1996, Cancer Treat. Res. 87: 39-62). Both P-gp and MRP belong to a large family of ATP trafficking proteins that are evolutionary conserved and mediate the transport of numerous ligand ranging from ions to large polypeptides (Higgins et al., 1992, Ann. Rev. Cell Biol. 8: 67-113). In tumor cell lines, P-gp and MRP reduce the accumulation of drugs via an energy dependent drug efflux mechanism (Shapiro et al., 1994, J. Biol. Chem. 269(5): 3745-3754; Doige et al., 1992, Biochim. Biophys. Acta 1109: 161-171).

P-gp and MRP expression has been detected in normal tissues and is thought to mediate the transport of normal cell metabolites and xenobiotics (Cordon-Cardo et al., 1990, J. Histochem. Cytochem. 38(9): 1277-1287; Bradley et al., 1990, J. Cell. Physiol. 145: 398-408; Thorgeirsson et al., 1987, Science 236: 1120-1122; Thiebaut et al., 1987, Proc. Nat. Acad. Sc. USA 84: 7735-7738). Consistent with these speculations, inactivation of both alleles of P-gp from the mouse genome has led to the accumulation of drugs and natural products in many organs where P-gp is highly expressed (Schinkel et al., 1997, Proc. Nat. Acad. Sc. 94(8): 4028-4033; Schinkel et al., 1994, Cell 77: 491-502). High levels of P-gp has been shown in 20 - 70% of tumors from different cancers (Tishler et al., 1992, J. Neurosurgery 76: 507-512; Abe et al., 1994, Japan J. Cancer Res. 85(5): 536-541; Baker et al., 1989, ?

: 87-97; Belloni et al., 1989, Cancer and Metastasis Rev. 8: 353-389; Charpin et al., 1994, J. Nat. Cancer Inst. 86(20): 1539-1545; Fojo et al., 1987, Proc. Nat. Acad. Sc. 84: 265-269; Henson et al., 1992, J. Neuro-Oncology 14: 37-43; Hijazi et al., 1994, Am. J. Clin. Pathol. 102(1): 61-67; Mattern et al., 1994,

Anticancer Res. 14(2A): 417-419), and in some tumors (e.g. haemepoitic tumors and childhood malignancies) P-gp expression has been shown to predict clinical drug resistance and long term survival (Nooter et al., 1994, Leukemia Research 18(4): 233-243; Chan et al., 1995, Hematology - Oncology Clinics of North America 9(2): 275-318; Chan et al., 1991, New Engl. J. Med., 325:1608-1614). However, the lack of P-gp expression in other multidrug resistant tumors indicates other cellular changes that confer resistance to anticancer drugs (Lee et al., 1997, J. Cell. Biochem. 65(4): 513-526; Baggetto, Bull. Cancer. 84(4): 385-390; Linn et al., 1994, J. Clin. Oncol. 12(4): 812-819; Lonn et al., 1994, Intern. J. Cancer 58(1): 40-45; Sognier et al., 1994, Biochem.l Pharmacol. 48(2): 391-401). Some of the cellular changes identified in drug resistant cells include the overexpression of MRP (Zaman et al., 1994, Proc. Nat.l Acad. Sc. USA 91(19): 8822-8826), alterations in glutathione-S-transferase activity or GSH levels (Tew, 1994, Cancer Res. 54(15): 4313-4320), reduction in topoisomerase II levels or activity (Frelich et al., 1995, J. Biol. Chem. 270(15): 21429-21432), overexpression of LRP (the Lung Resistance Protein, a the component of human vaults) (Scheffer et al., 1995, Nature Med. 1(16): 578-578) and alteration in functions or levels of proteins mediating apoptosis or programmed cell death (Lowe et al., 1993, Cell 74(6): 957-967; Lowe et al., 1994, Science 266(5186): 807-810).

There thus remains a need to identify other cellular changes that confer drug resistance.

The overexpression of a 40 kDa protein (P-40) alone or together with P-gp or MRP in MDR cell lines (Wang et al., 1997, Biochem. Biophys. Research Communications. 236(2): 483-488) has been previously reported. However, further studies were required to demonstrate a direct role for P-40, if any, in drug metabolism and multidrug resistance (MDR). Indeed, it was disclosed that it was unknown and unclear whether P-40 could modulate a MDR phenotype directly or indirectly (Wang et al., 1997, *supra*).

There thus remains a need to determine whether P-40 is indeed directly implicated in MDR. In the affirmative, there also remains a need to identify the molecular determinant of this MDR, in the form of a nucleic acid and for protein in order to open the way for the obtention of diagnostic, therapeutic and research tools in the field of multidrug resistance.

The present invention seeks to meet these and other needs.

SUMMARY OF THE INVENTION

The invention concerns the demonstration that P-40 has a direct role in multidrug resistance.

Further, the invention relates to the identification of P-40 as a member of the MDR gene family and to the identification of P-40/Annexin I related genes as members of this broadened MDR gene family.

In addition, the invention relates to the identification of Annexin II and IV as potential MDR determinants. Thus, the invention relates to the identifications of Annexins (I to XI, also referred thereto herein P-40 and P-40 homolgs) as potential members of the MDR gene family. Broadly therefore, the present invention also relates to the identification of Annexin-based MDR in cells.

The present invention further relates to the isolated nucleic acid molecules encoding P-40 or fragment thereof and to the identification of P-40 as Annexin I.

The invention in addition relates to purified P-40 polypeptides, homologs thereof, or epitope binding portions thereof and the use thereof in multidrug resistance. The invention also provides a specific detection method for P-40 nucleic acids encoding P-40 proteins or homologs thereof, polypeptides or fragments thereof in a sample.

In addition, the invention provides a recombinant nucleic acid molecule comprising P-40 (or homologs thereof) operationally linked to a

promoter, efficient in initiating transcription thereof in a host cell as well as to such a host cell.

As well, the invention provides a non-human organism containing the nucleic acid molecule mentioned above. Further, the invention provides an antisense P-40 or P-40-related (Annexin I-related) nucleic acid molecule.

The invention further provides an antibody having specific binding affinity towards P-40, P-40 homologs or an epitope-containing-region thereof. In one embodiment, the antibody is a monoclonal. The invention also provides the hybridoma producing the monoclonal antibody.

The invention also seeks to provide a method for the detection of P-40 or P-40 protein homologs or portions thereof in a sample. In one embodiment, such a method is quantitative.

Furthermore, the invention seeks to provide a diagnostic kit comprising a first contained means containing the above-mentioned antibody, and second container means containing a conjugate comprising a binding partner of the monoclonal antibody and a label.

The invention seeks to provide diagnostic methods for human disease and particularly for cancer and the multidrug resistance of cancer cells. Preferably, a method for evaluating the predisposition of a cancer tumor to be and/or become multidrug resistance is provided herein.

The invention further seeks to provide therapeutic methods involving the P-40 nucleic acid homologs, variants or parts thereof, antisense thereof, P-40 protein, P-40 protein homologs or P-40 antibodies.

The present invention also relates to a kit comprising the oligonucleotide primers or agents or ligands of the present invention which are specific to annexins and more particularly to annexin I.

For example, a compartmentalized kit in accordance with the present invention includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers

or strips of plastic or paper. Such containers allow the efficient transfer of reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated ant the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample (DNA or cells), a container which contains the primers used in the assay (or antibodies or other ligands), containers which contain enzymes, containers which contain wash reagents, and containers which contain the reagents used to detect the extension products (or reagents to detect the antibodies or ligands).

It shall be understood that in certain situations it might be beneficial to render a cell MDR by providing thereinto at least one nucleic acid encoding an Annexin (or at least one Annexin protein) so as to give this cell a growing advantage with respect to wild-type cells in certain growth conditions (i.e. presence of drug).

It is shown herein that P-40 is Annexin I. Annexin I is a member of a large family of Ca2+ phospholipid binding proteins [for review see (Raynal et al., 1994, Biochim. Biophys. Acta. 1197: 63-93), implicated in several cellular mechanisms including intracellular membrane vascular trafficking and exocytosis process (Creutz, 1992, Science. 258: 924-93; 1Lin et al., 1992, Cell 70: 283-291; Creutz et al., 1992, J. Cell Science. 103: 1177-1192). However, Annexin I has not been previously implicated in drug resistance or suggested to be implicated hereinto. The inventors are then the first to show the role of Annexin I in expression of drug resistance to anticancer drugs. Thus, the present invention is the first to show an Annexin-based mutlidrug resistance pathway in cells.

Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols

as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission.

The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

As used herein, "isolated nucleic acid molecule", refers to a polymer of nucleotides. Non-limiting examples thereof include DNA and RNA molecules purified from their natural environment.

The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering.

The term "DNA segment", is used herein, to refer to a DNA molecule comprising a linear stretch or sequence of nucleotides. This sequence when read in accordance with the genetic code, can encode a linear stretch or sequence of amino acids which can be referred to as a polypeptide, protein, protein fragment and the like.

The terminology "amplification pair" refers herein to a pair of oligonucleotides (oligos) of the present invention, which are selected to be used together in amplifying a selected nucleic acid sequence by one of a number of types of amplification processes, preferably a polymerase chain reaction. Other types of amplification processes include ligase chain reaction, strand displacement amplification, or nucleic acid sequence-based amplification, as explained in greater detail below. As commonly known in the art, the oligos are designed to bind to a complementary sequence under selected conditions.

The nucleic acid (i.e. DNA or RNA) for practising the present invention may be obtained according to well known methods.

Oligonucleotide probes or primers of the present invention may be of any suitable length, depending on the particular assay format and the particular needs and targeted genomes employed. In general, the oligonucleotide probes or primers are at least 12 nucleotides in length,

preferably between 15 and 24 molecules, and they may be adapted to be especially suited to a chosen nucleic acid amplification system. As commonly known in the art, the oligonucleotide probes and primers can be designed by taking into consideration the melting point of hydrizidation thereof with its targeted sequence (see below and in Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, CSH Laboratories; Ausubel et al., 1989, in Current Protocols in Molecular Biology, John Wiley & Sons Inc., N.Y.).

"Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having complementary base sequences, which under appropriate conditions will form a thermodynamically favored double-stranded structure. Examples of hybridization conditions can be found in the two laboratory manuals referred above (Sambrook et al., 1989, and Ausubel et al., 1989) and are commonly known in the art. In the case of a hybridization to a nitrocellulose filter, as for example in the well known Southern blotting procedure, a nitrocellulose filter can be incubated overnight at 65°C with a labeled probe in a solution containing 50% formamide, high salt (5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 µg/ml denatured carried DNA (i.e. salmon sperm DNA). The non-specifically binding probe can then be washed off the filter by several washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in view of the desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (Tm) of the DNA hybrid. Of course, RNA-DNA hybrids can also be formed and detected. In such cases, the conditions of hybridization and washing can be adapted according to well known methods by the person of ordinary skill. Stringent conditions will be preferably used (Sambrook et al.,1989, supra).

Probes of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and α-nucleotides and the

like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic acid molecule. Acids Res., 14:5019. Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA.

The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less prepared, labeled proteins could also be used to detect a particular nucleic acid sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like.

Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the sensitivity of the detection. Furthermore, it enables automation. Probes can be labeled according to numerous well known methods (Sambrook et al., 1989, supra). Non-limiting examples of labels include ^{3}H, ^{14}C, ^{32}P, and ^{35}S. Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma ^{32}P ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* in the presence of radioactive dNTP (i.e. uniformly labeled DNA probe using random oligonucleotide primers in low-melt gels), using the SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

As used herein, "oligonucleotides" or "oligos" define a molecule having two or more nucleotides (ribo or deoxyribonucleotides). The size of the oligo will be dictated by the particular situation and ultimately on the particular use thereof and adapted accordingly by the person of ordinary skill. An oligonucleotide can be synthetised chemically or derived by cloning according to well known methods.

As used herein, a "primer" defines an oligonucleotide which is capable of annealing to a target sequence, thereby creating a double stranded region which can serve as an initiation point for DNA synthesis under suitable conditions.

Amplification of a selected, or target, nucleic acid sequence may be carried out by a number of suitable methods. See generally Kwoh et al., 1990, Am. Biotechnol. Lab. 8:14-25. Numerous amplification techniques have been described and can be readily adapted to suit particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-based amplification, the Qβ replicase system and NASBA (Kwoh et al., 1989, Proc. Natl. Acad. Sci. USA 86, 1173-1177; Lizardi et al., 1988, Biotechnology 6:1197-1202; Malek et al., 1994, Methods Mol. Biol., 28:253-260; and Sambrook et al., 1989, supra). Preferably, amplification will be carried out using PCR.

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer which is synthesized is complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to

hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analysed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization following EtBr staining of the DNA following gel electrophores, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990).

Ligase chain reaction (LCR) is carried out in accordance with known techniques (Weiss, 1991, Science 254:1292). Adaptation of the protocol to meet the desired needs can be carried out by a person of ordinary skill. Strand displacement amplification (SDA) is also carried out in accordance with known techniques or adaptations thereof to meet the particular needs (Walker et al., 1992, Proc. Natl. Acad. Sci. USA 89:392-396; and ibid., 1992, Nucleic Acids Res. 20:1691-1696.

As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise the a specific polypeptide or protein. It will readily recognized by the person of ordinary skill, that the nucleic acid sequence of the present invention can be incorporated into anyone of numerous established kit formats which are well known in the art.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

The term "expression" defines the process by which a structural gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or more.

The terminology "expression vector" defines a vector or vehicle as described above but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being operably linked to control elements or sequences.

Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of a sequence whether an nucleic acid or amino acid sequence, a molecule that retains a biological activity (either function or structural) that is substantially similar to that of the original sequence. This functional derivative or equivalent may be a natural derivatives or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid as chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophylicity and the like. The term "functional derivatives" is intended to

include "fragments", "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the present invention.

Thus, the term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention.

The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. all these methods are well known in the art.

As used herein, "chemical derivatives" is meant to cover additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic of the derivative (i.e. solubility, absorption, half life and the like, decrease of toxicity). Such moieties are exemplified in Remington's Pharmaceutical Sciences (1980). Methods of coupling these chemical-physical moieties to a polypeptide are well known in the art.

The term "allele" defines an alternative form of a gene which occupies a given locus on a chromosome.

As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. As well known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotide. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. The result of a mutations of nucleic acid molecule is a mutant nucleic acid molecule. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a "purified protein" has been purified to a level not found in nature. A "substantially pure" molecule is a molecule that is lacking in all other cellular components.

While the property of a host cell to become MDR is demonstrated with human P-40/Annexin I other Annexins (II-XI), non-human Annexins, other biologically functional genes/cDNA-related to Annexins can also be used in the context of the present invention. For example, Mouse Annexin I could be used in some embodiments of the present invention as will be recognized by a person of ordinary skill.

Annexins are part of a gene family of multifunctional calcium- and phospholipid - binding protein (for a review see Raynald et al., 1994, Biochem., Biophys. Acta., 1197:45-62). They have been described in many organisms from mammals, to molds and even plants, and their similar functional properties in Ca^{2+} and phospholipid are explainable by their common primary structure (Raynald et al., 1994, supra). Indeed, some of the Annexins are thought to have originated from a common ancestor (Raynald et al., 1994, supra). Moreover, the family of Annexin genes shows very significant identity between human, rat, and mouse homologs (Raynald et al., 1994, supra). It will be clear to the person of ordinary skill that the present invention is not to be limited to human Annexins as homologs having the biological function of Annexin-based MDR can be used within the context of the present invention.

Furthermore, since Annexins are found in diverse evolutionary distant organisms such as plants, yeasts, and parasites (Raynald et al., 1994, supra), the present invention has very broad implications. For example, the present invention opens the way to use Annexins from diverse organisms, such as yeast (i.e. Candida albicans) and parasites as therapeutic targets. Antifungal drugs, for example, could be identified by using yeast Annexins as therapeutic targets.

The presence of Annexins in plants could find utility in the development of specific crop resistance, by for example, increasing the expression level of at least one Annexin.

In accordance with yet another aspect of the present invention, there is provided a method of reducing Annexin-based MDR in a cell

or animal, comprising the step of administering a therapeutically effective amount of a pharmaceutical composition according to the instant invention.

For pharmaceutical administration, the said polypeptide may be incorporated into preparations in either liquid or solid forms using carriers and excipients conventionally employed in the pharmaceutical art, optionally in combination with further active ingredients. The preparation may, for example, be applied orally, parenterally, enterally or preferably topically. Preferred forms include, for example, solutions, emulsions, gels, sprays, lotions, ointments, creams or powders.

One of ordinary skill can readily determine the amounts of Annexin-based MDR reducing agent to be administered. It is apparent that the dosage will be dependent on the particular treatment used. It should also be clear that the dosage should be chosen to display the biological activity without causing adverse effects. It will be understood that age, sex, type of disease, of formulation and other variables known to the person of ordinary skill will affect determination of the dosage to be used.

The pharmaceutically acceptable carriers and excipients are well known in the art. A representative textbook thereon is Remington's Pharmaceutical Sciences, 1980, 16th Ed., Mack Eds.

Advantageously the compositions may be formulated as dosage units, each unit being adapted to supply a fixed dose of active ingredient. The total daily dose may, of course, be varied depending on the subject treated and the particular use of the composition. This can obviously be adapted by the treating professional.

In general, techniques for preparing antibodies (including monoclonal metabolism and hybridomas) and for detecting antigenes using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology", Elsevier Science Publisher, Amsterdam, The Netherlands) and in Harlow et al., 1988 (in: Antibody - A Laboratory Manual, CSH Laboratories).

The present invention opens the way to the identification of agonists and antagonists of Annexins with respect to their role in MDR. An assay for Annexin-based MDR activity in cells can be used to assess the effect of agents on Annexins function in drug resistance and therefore identify such agonists or antagonists. Non-limiting examples of such agents include nucleic acid molecules, peptides, antibodies, carbohydrates, or other pharmaceutical agents.

The present invention also provides polyclonal, monclonal antibodies, or humanized versions thereof, chimeric antibodies and the like which inhibit or neutralize their respective Annexin targetted antigens *in vivo* and/or specific thereto.

Treatments comprise parenteral administration of multiple or single doses of the above listed antibodies and derivatives thereof. The dosage will be varied by the practitioning professional depending on the usual parameters such as pharmacodynamic characteristics the route of administration, recipient's characteristics, symptoms and/or disease thereof and the like. A daily dosage of active ingredient can be for example about 0.1 to 100 mg/kg of body weight, ordinarily 0.5 - 50 and preferably 1-10 mg/kg per day, i.e. divided doses ranging from 1-6 times per day or alternatively in sustained release form.

The non-human animals of the present invention having a transgenic interruption or alteration of the Annexin endogenous gene(s) (knock-out animal) and/or into the genome in which transgenes directing expression of Annexin(s) has been introduced include vertebrates such as rodents, non-human primates, amphibians, reptiles and the like. These animals are prepared in accordance with known methods. The same applies to transgenic plants.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1A shows a restriction map of the gene encoding P-40. The map for the 1.4 kb fragment isolated from λgt11 expression library with IPM96 monoclonal antibody. The restriction enzyme sites are indicated by the arrows. The solid bar indicates the coding sequence for P-40 or Annexin I. The thin lines to the left and right of the solid bar indicate the 5' and 3' non-coding regions. Figure 1B shows the nucleic acid sequence of P-40/Annexin I. Figure 1C shows the amino acid sequence of P-40/Annexin I. Figure 1D shows the nucleic acid sequence of both strands of P-40/Annexin I and hence shows examples of antisense nucleic acid molecules which can be used in accordance with the present invention.

Figure 2 shows the *in vitro* expression of P-40. The *in vitro* expression of P-40 gene was performed using the T7 promoter directed transcription in TA PCRII cloning vector. Figure 2a shows *in vitro* transcribed and translated mixes containing PCRII vector only immunoprecipitated with IPM96 mAb or PCRII plus 1.4 kb insert immunoprecipitated with IPM96 mAb or an irrelevant IgG2b (lanes 2 and 3, respectively). Lanes 1-4 of figure 2b show the same samples as in lanes 1 and 2 of Fig. 2a but transferred to nitrocellulose membrane and probed with a specific antibody to P-40 (IPM96 mAb ; Wang et al., 1997, Biochem. Biophys. Res. Com. 236(2): 483-488) or an irrelevant IgG2b, respectively.

Figure 3 shows the analysis of protein and mRNA levels in drug sensitive and resistant cells. In Fig. 3a, total cell extract from drug sensitive (MCF-7, SKOV3 and H69) and drug resistant human MDR cell lines (MCF-/Adr, SKOV/VLB$^{1.0}$ and H69/AR) were fractionated on SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with IPM96 monoclonal antibody. P-40 (or annexin I) is seen in extracts from drug resistant

level of P-40 is detected in SKOV3 drug sensitive cells but not in MCF-7 or H69 cells. For mRNA levels in the same cell lines, total RNAs were resolved on agarose gel and transferred to nylon membrane and probed with ^{32}P-dATP labelled 1.4 kb fragment encoding for P-40 (or annexin I) and actin (Fig. 3b). Figure 3b shows that the expression of a 1.6 kb mRNA in drug sensitive and resistant MDR cells correlates with levels of P-40 (or annexin I) in the same cell lines.

Figure 4 shows the post-translational modification of P-40 or Annexin I in MCF-7/AR cells. Cells were metabolically labeled with ^{35}Smethionine or ^{32}P inorganic phosphate and radiolabeled protein were immunoprecipitated with an irrelevant IgG2b or IPM96 mAb (lanes 1 and 3 or 2 and 4, respectively).

Figure 5 shows the expression of Annexins I, II, IV and VI in drug sensitive and resistant cells. Total cell lysates from drug sensitive (MCF-7, H69, SKOV3 and AuxB1) and resistant (MCF-7/Adr, H69/AR, SKOV3/VLB$^{1.0}$, SKOV3/VLB0.06 and CH^{r}C5) or revertant (H69/PR) were resolved on SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membranes was then probed with anti-Annexin I, II, IV or VI monoclonal antibodies.

Figure 6 shows the drug sensitivity assays for P-40 transient transfectants. The level of P-40 (Annexin I) expression in MCF-7 transiently transfected cells was determined by Western blotting (figure 6a). MCF-7 cells transfected with pCDNA3 vector without or with P-40 (Annexin I) gene were incubated in the absence and presence of increasing concentrations of Taxol (figure 6b) or adriamycin (doxorubicin) (figure 6c).

Figure 7 shows the P-40 (or annexin I) construct pCIN4P-40. The full length P-40 (or annexin I) cDNA was cloned into the Not-1 site of eukaryotic expression vector pCIN4 in both sense and antisense orientations. This places (or operately links to the promoter) the P-40 (or annexin I) adjacent to the viral CMV promoter, which directs its expression. The neomycin phosphotransferase gene is also present on the construct with its expression

being driven by the same promoter element located ahead of multi-cloning sites (indicated in the map), thus providing G418 resistance to cells containing a construct. To facilitate translation of a second open reading frame (neomycin phosphotransferase), the encyphalomyocarditis virus internal ribosome entry sites (IRES) has been inserted into the expression cassette immediately upstream of the start codon of the neomycin phosphotransferase ORF. Intervening sequences (IVS) accompany the transcription complex (P-40cDNA and neomycin phosphotransferase gene) for maximal expression of mature transcripts. The pCIN4P-40 vector can be used to generate stable transfectants.

Figure 8 shows the expression of P-40 (or annexin I) in MCF-7 stable transfectants. The expression level of P-40 was determined by Western blot and indirect immunofluorescence. Figure 8a shows equal number of cells from MCF-7 transfected with vector alone (lane 1) or vector plus P-40cDNA (P-40-MCF-7) (lane 2) were lysed and the total lysates were separated by 10% SDS PAGE and blotted onto PVDF membrane. The blot was probed with IPM96 monoclonal antibody. Figure 8b shows immunohistochemical staining of MCF-7 cells transfected with vector alone or vector plus P-40cDNA. The expression of P-40 (or annexin I) is stable transfectants (P-40-MCF-7) was determined using the cytospins probed with IPM96 mAb and FITC conjugated goat anti-mouse secondary antidoby as described in Materials and Methods. Figure 8b shows the expression of P-40/annexin I by immunofluorescence analysis using the IPM96 mAb.

Figure 9 shows the effects of anticancer drugs on MCF-7 cells stably expressing P-40 (or annexin I). MCF-cells transfected with P-40 cDNA or vector only, were incubated in the absence and presence of increasing concentration of adriamycin (doxorubicin), actinomycin D, Taxol and cisplatin. The sensitivity of the cells to the drugs was measured by the tetrazolium salt based assay as described in Materials and Methods. Éxperiments were performed in triplicates.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

MATERIALS AND METHODS

Cell culture and metabolic labelling

Drug sensitive (MCF-7, H69, SKOV3 and HL60) and resistant (MCF-7/Adr, H69/Adr, SKOV3/VLB$^{1.0}$ and HL60/AR) cells were grown in the absence of antibiotics in α-MEM or RPMI-1640 media supplemented with 5% to 15% fetal calf serum (Hyclon. Inc.) as previously described (Mirski et al., 1987, Cancer Res. 47: 2594-2598; McGrath et al., 1987, Biochem. Biophys. Res. Com. 145(3): 1171-1176; Bradley et al., 1989, Cancer Res. 49: 2790-2796; Batist et al., 1986, J. Biol. Chem. 261(33): 15544-15549). Briefly, cells were grown at 37°C in humid atmosphere of 5% CO2 and 95% air. Cells were passaged when 70-80% confluent for adherent cells and 1X106 cell/ml for cells in suspension. Drug resistant cells were grown continuously with the appropriate concentrations of cytotoxic drugs 24 hours following subculturing. All cells were examined for *Mycoplasma* contamination using the *Mycoplasma* PCR method (Strategen Inc. San Diego, CA). For metabolic labeling of cells, MCF-7/Adr cells at 70-80% confluency were metabolically labeled with [^{35}S] methionine (100 µCi/ml; 1000 Ci/mmol; Amersham Life Sciences, Inc.) or Carrier free ^{32}P inorganic phosphate (8 mCi/ml; Amersham Life Sciences, Inc.) for 3-4 hours at 37° C in methionine- or phosphate-free α-MEM. Cells were lysed and the cell lysates were immunoprecipitated with IgG_{2b}, or IPM96 mAb.

Screening an expression library with IPM96 monoclonal antibody

A 5' stretch cDNA expression library of HeLa cells constructed into λgt11 vector was obtained from Clontech (Palo Alto, CA). About $1X10^6$ plaque forming units were plated using *Escherichia coli* Y1090 as host and screened with IPM96 monoclonal antibody. Briefly, plates containing phage plaques were incubated at 42°C for 4 hours and then overlayed with a dried nitrocellulose filter saturated in 10 mM X-gal. The plates were continuously incubated at 37°C for another 3 hours and the filters were rinsed with TBST buffer (50 mM Tris-HCL pH 7.9, 150 mM NaCl, 0.05% Tween-20) and incubated in TBST containing 5% of skin milk for 30 minutes with gentle agitation. The nitrocellulose disks were incubated with TBST buffer containing 2 µg/ml of Protein G purified IPM96 monoclonal antibody. The reactive plaques were detected with a second goat anti-mouse IgG conjugated to horseradish peroxidase and visualized chemiluminescence using Amersham ECL kit. The immunoreactive plaques were verified by duplicate lifts and purified by subsequent rounds of screening using decreased plaque density. Immunoreactive plaques were eluted in ddH2O and utilized as template for PCR directed by the 3' and 5' insert screening amplimer sequence of λgt-11 and the fragment from PCR was cloned into PCR II vector (InVitrogen Inc.) following standard procedures (Sambrook et al., 1989, Molecular cloning: A laboratory Manual. Cold Spring Harber Laboratory Press, Cold Spring Harbor, N.Y.)

Nucleotide Sequencing and Computer Sequence Analysis

The cDNA clones of the present invention were sequenced by the dideoxy chain termination method using M13 universal primer and sequence specific primers via the automated DNA sequencing service at the Sheldon Biotech Centre at McGill University. Both strands of two different pick clones were completely sequenced. Computer analysis of the DNA and protein sequence was done using the Blast DNA search programs.

Northern blot and DNA slot blot analyses

For Northern blot, total RNAs from drug sensitive and resistant cells were extracted with the Trazol solution (GibcoBRL, Gaithersburg, U.S.A). Approximately 10 µg of RNA from each cell line was run on a 1% formaldehyde-denatured agarose gel and transferred to HybondsTM-N nylon membrane (Amersham, Oakvill, Ont.) by pressure blotting with 20X SSC (1X = 140 mM NaCl, 32 mM Sodium Citrate, pH 7.4). The blot was incubated in the presence of 32P labeled P-40 cDNA probe by nick translation or actin gene in 50% formamid, 2.5X Denhardtís solution, 25 mg/ml denatured Salmon sperm DNA, 1% SDS and 1.25X SSPE overnight. The blot was washed in low stringency wash with 0.5X SSC for 30 minute at room temperature. A higher stringency wash was applied only if it was necessary. RNA integrity and equal loading was assessed in all cases by hybridization with actin or rRNA probes. Quantification of radioactive signals was carried out by scanning the resultant autoradiography and analysis with NIH imaging software (Wayne, 1992, NIH Imaging Software).

For slot blotting, genomic DNA from MCF-7 and MCF-7/Adr cells was prepared as described (Sambrook et al., 1989, *supra)* and 10 µg DNA from each sample was denatured by 1 M NaOH. Following 10 minute boiling at 100°C. The denatured DNA was diluted serially loaded onto Nylon membrane by using Manifold Slot Blot apparatus (Pharmacia Inc.). The blot was then hybridized with a 32P-labeled probe at 42°C in 50% formamide, 2.5XDenhardt's solution, 25 mg/ml denatured summon sperm DNA, 1% SDS and 1.25X SSPE overnight. The highest stringent wash of the blot was 0.5X SSC with 1% SDS at 65°C for 30 minutes.

In vitro Translation and Immunoprecipitation

In vitro transcription and translation reactions were carried out using a rabbit reticulocyte lysate (Promaga Corporation. Madison, WI) and 35S-methionine (Dupont/NEW, Mississauga, Ont.) according to the manufacturer's protocol. Briefly, cDNA clone encoding full length P-40 was

cloned into NotI and XhoI sites of a pCDNA3 expression vector containing T7 and SP6 promotor sequences (In Vitrogene, Inc.). The pCDNA3 with and without P-40 insert were added to a coupled reticulocyte lysate transcription and translation system in the presence of [35S]-methionine. Following a 2 hour incubation at 30°C, *in vitro* synthesized proteins were immunoprecipitated with IPM96 monoclonal antibody as previously described (Georges et al., 1991, J. Cell. Physiol. 148: 479-484; Wang et al., 1997, *supra*).

DNA Transfection

MCF-7 cells were transfected with the pCDNA3 or pCIN4 vectors or vector containing P-40 coding sequence using lipofectAMINE (Gibco, Burlington, Ontario, Canada) according to the manufacturer's procedure. Briefly, 4 X 10^5 cells in a 60 mm plate with 5 ml of serum-free minimal essential medium were overlaid with 200 µl of serum-free essential medium containing 5 µg of supercoiled DNA mixed with 10 µl of lipofectAMINE. After 5 hour incubation, the medium was replaced with 5 ml of minimal essential medium (MEM) supplemented with 10% fatal calf serum (Hyclone Laboratories) and cells were further cultured at 37°C for 24 - 48 hours. For the transient transfection, cells were collected and the expression of P-40 was detected by western blot and immuno- fluorescence. For stable transfectants, G418 was added to the cells at 1 mg/ml and continuously selected for another two-three weeks. The individual transfectant clones of MCF-7 cells were obtained by the limited dilution under the G418 treatment (i.e. pCIN4-P40 and PCIN4 stably expressing cells). A population exhibiting highly expressed P-40, designated BM-1, and a population of the cells transfected with pCDNA3 alone, designated AM-1 were expanded for further analysis by Western blot or immunoprecipitation. A population of cells exhibiting high stable expression of P-40 and a population of the cells transfected with pCIN4 vector alone were characterized by Western blotting and immunofluorescence.

Immunofluorescence Staining of Cells

Cells were washed with PBS and smeared onto glass slides by brief centrifugation at 1,500 rpm. The cytospins were air-dried and fixed for 10 minutes in ice-cold acetone. Cells were rinsed twice with PBS and incubated in 1% bovine serum albumin (BSA)/PBS for 30 minutes at room temperature. Slides were incubated with the IPM96 mAb (5 µg/ml 1% BSA/PBS) for 30 minutes followed by three two minutes rinses with PBS. FITC-conjugated goat-anti-mouse IgG (1:50 dilution) was added to slides and allowed to incubate for 30 minutes. After several washes, slides were mounted in PBS containing 50% glycerol and examined with a Nikon UFX-DX fluorescent microscope fitted with a 60X oil immersion objective. Photographs were taken with Kodak Tri-X pan film (400 ASA) at 800X magnification.

Drug Sensitivity Assay

Transient transfectants were harvested 48 hours later and aliquoted at $7.5X10^3$ cells per well of a 96-well plate. Drugs were added to cells 24 hours later and incubated for another 48 hours at 37°C. The assay was developed by adding 50 µl (5 mg/ml) of an MTT dye and allowed to incubate for four hours at 37°C as previously described (Pouliot et al., 1997, Biochem. Pharmacol. 53(1): 17-25) .

SDS-PAGE and Western Blotting

Total cell lysates cell cultures or from transcription/translation reaction mixture were mixed with equal volumes of sample buffer containing SDS and the denatured proteins were resolved on 10% polyacrylamide gels according to the method of Laemmeli (Laemmli et al., 1970, Nature 227(259): 680-685). For acrylamide gels containing 35S-methionine labeled proteins, gels were fixed in 50% methanol/water and soaked in AmplifyTM (Amersham, Oakvill , Ont.) for 30 minutes prior to drying and exposing to Kodak film at -70°C. For Western blot analysis, proteins were transferred to nitrocellulose membrane

for 1 hour at 50 volts according to the method of Towbin et al., (Towbin et al., 1979, Proc. National Acad. of Sciences of the United States of America 76(9): 4350-4354). The nitrocellulose membrane was blocked with 5% skin milk/PBS and incubated overnight at 4°C with 1 µg/ml of IPM96 monoclonal antibody. The immunoreactive proteins were detected with horseradish peroxidase conjugated goat anti-mouse antibody and visualized by chemoluminence using Amersham ECL kit (Amersham, Oakvill , Ont.).

Cytotoxicity assays

The chemosensitivity patterns of stably transfected MCF-7 cells with pCIN4 vector alone or full length P-40 cDNA were determined by a tetrazolium salt-based microplate assay as described previously (Pouliot et al., 1997). Briefly, 100 μl aliquots of cells were plated into 96-well plates at 5000 cells per well. The cells were then incubated at 37° C for 24 hours before the addition of increasing concentration of different cytotoxic agents. Following a 72 hour incubation with different anticancer agents, 3-(4,5-Demethylthiazol-2-yl)-,5-dyphenyl-tetrazolium bromide (Sigma) was added to each well of the plates at a final concentration of 2.5 mg/ml. After 4 hour incubation, cells were solubilized by the addition of 50 μl of 10% Triton X-100 in 0.01 N HCl. The 96-well plates were heated in the microwave oven for 1 minute at the minimal power setting, and 10 μl of 100% ethanol was added to disperse the bubbles formed during pipeting. Plates were read at 570 nm using an ELISA microplate reader. The effects of drugs on the viability of cells were expressed as the mean ± SD of two to three independent experiments in which triplicates were assayed.

RESULTS

Isolation and characterization of P-40 cDNA clones

In a previous report (Wang et al., 1997, *supra*) ,we had demonstrated the overexpression of a 40 kDa protein (P-40) in several MDR cell lines alone or together with P-gp and MRP. In this study, we have used the

monoclonal antibody IPM96 which binds specifically to P-40 to screen a cDNA expression library made from HeLa cells. A total of 50,000 plaques were screened and several positive plaques were identified following the initial screening. Of the latter positive plaques two positive clones were obtained after sequential plaque purification and both inserts were isolated by PCR (see Material and Methods). Both positive clones encoded for a 1.6 kb fragments that were subsequently cloned into a T/A PCRII vector. Sequence analysis of both clones showed an open reading frame of 346 amino acids which is consistent with the molecular mass of the protein (38.7 kDa versus 40 kDa) (figure 1A). Comparison of P-40 nucleotide and amino acid sequences (figures 1B and 1C) to other sequences in the DNA data bank, using the DNA search programs TBlast, showed it to be identical to Annexin I sequence (Wallner et al., 1986, Nature 320: 77-81).

To confirm the identify of the isolated cDNA, a 1.4 kb encoding the full length of P-40 (Annexin I) was expressed *in vitro* using T7 promotor directed transcription-translation reticulocyte lysate with ^{35}S-methionine. Figure 2a shows the immuno- precipitation of proteins with IPM96 mAb from an *in vitro* expression reactions containing vector only (lane 1) or vector plus 1.4 kb insert (lane 2). As a control for the IPM96 antibody, an irrelevant IgG2b was used to immunoprecipitate proteins from a reaction mix containing vector plus the 1.4 kb insert (figure 2a, lane 3). The results of figure 2a show a ^{35}S-methionine labeled 40 kDa protein immunoprecipitated with IPM96 mAb but not with an irrelevant IgG2b (lanes 2 and 3, respectively). Figure 2b, also shows Western blotting of the proteins identical to those in lanes 1 and 2 of figure 2a, but probed with IPM96 mAb (lanes 1 and 2) or an irrelevant IgG2b (lanes 3 and 4). Taken together, these results confirm the identity of the 1.4 kb fragment as the gene encoding P-40 or Annexin I.

To determine if the amino acid sequence of P-40 (or Annexin I) cloned from HeLa cells is similar or different from that found in MCF-7/AR cells, Annexin I was cloned from MCF-7/AR cells by reverse PCR

using 5' and 3' primers encoding Annexin I. Sequence analysis of Annexin I clones from MCF-7/AR cells revealed no differences from that isolated from HeLa cells cDNA expression library (data not shown).

Northern blot analyses

In an earlier study (Wang et al., 1997, *supra*) , the levels of P-40 was compared between drug sensitive and resistant cell lines. Figure 3a shows a Western blot analysis of total cell lysates from drug sensitive (MCF-7, SKOV3 and H69) and their resistant (MCF-7/AR, SKOV3/VLB$^{1.0}$ and H69/AR) cells probed with IPM96 mAb. The results of the latter Western blot analysis shows clearly the increase in P-40 expression in resistant cells relative to the parental drug sensitive cells. The SKOV3 cells show lower levels of P-40 than the resistant SKOV3/VLB$^{1.0}$. However, it is interesting that the SKOV3 cell line was derived from a patient with ovarian tumor that was considered to be clinically resistant Cis-platinum and adriamycin [(ogh et al., 1975, Plenum Press, New York : 155-159). To determine if the increase in P-40 or Annexin I protein expression in the above MDR cells is due to an increase in mRNA levels, Northern blot analysis were performed with total RNA extracted from drug sensitive (MCF-7, SKOV3 and H69) and resistant (MCF-7/AR, SKOV3/VLB$^{1.0}$ and H69/AR) cells and blotted membrane probed with a 32P labeled 1.4 kb fragment. The results in figure 3b shows the 1.8 kb mRNA band in resistant MCF-7/AR and H69/AR but not in the parental drug sensitive cell lines, MCF-7 and H69/AR. The level of 1.8 kb mRNA hybridizing band was 4-fold higher drug resistant SKOV/VLB$^{1\,0}$ cells relative to drug sensitive SKOV-3 cells (figure 3b). Taken together, the Northern blot demonstrate clearly that the observed increase in P-40 or Annexin I cells is due to an increase in mRNA of P-40. Furthermore, the Northern blot results are consistent with the Western blot data, especially those relating to the protein and mRNA levels of P40 or Annexin I in SKOV3 versus SKOV3/VLB$^{1.0}$ cells (figure 3).

To determine if the above increase in mRNA levels in MDR cells relative to drug sensitive cells is at the transcriptional level or is due to gene

amplification, genomic DNA was isolated from the above cells lines and analyzed quantitatively using slot blot. The results (data not shown) show no gene amplification for P-40 or Annexin I between drug sensitive and resistant cells.

Post-translational modification of P-40 or Annexin I in resistant cells

Annexin I is a phosphoprotein phosphorylated at serine and tyrosine amino acids (Wang et al., 1994, Biochem. , 33: 276-282; Varticovski et al., 1988, Biochem., 27: 3682-3690). Further, it has been shown that phosphorylation of Annexin I at its N-terminal domain decreases its affinity for negatively charged phospholipids or membrane vesicle aggregation (Wang et al., 1994, Biochem., 33: 276-282]. Given the above results, it was of interest to examine the post-translational modification of P-40 or Annexin I in MDR cells. The results in figure 4 show immuno-precipitaton of P-40 or Annexin I with an irrelevant IgG2b or IPM96 mAb from MCF-7/AR cells that have been metabolically labeled with 35-methionine (lanes 1 and 2) or 32P inorganic phosphate (lanes 3 and 4). Interestingly, P-40 or Annexin I was not phosphorylated in MCF-7/AR cells. Similarly, it was not possible to demonstrate basal level of P-40 or Annexin I phosphorylation in the other MDR cells (data not shown).

Overexpression of Annexins II and IV in MDR cells

To determine if other members of the annexin family are similarly overexpressed in MDR cells relative to the parental drug sensitive cells, total cell proteins from drug sensitive (MCF-7, H69 and SKOV3) and resistant (MCF-7/AR, H69/AR and SKOV3/VLB$^{1.0}$) cells were separated by SDS-PAGE and transferred to nitrocellulose membrane. Figure 5 shows the results of the Western blots probed with anti-Annexin I, II, IV and VI monoclonal antibodies. The results of the Western blot probed with anti-Annexin I show similar results to the Western blot in figure 3a probed with IPM96, confirming the antigen specificity of IPM96 mAb towards to Annexin I. Furthermore, the expression of Annexin II and IV is also increased in MDR cells relative to the parental cell

lines, however to a lesser extend as that of Annexin I. However, unlike annexin I, both annexin II and IV are expressed, at lower levels, in drug sensitive cells (figure 5). Of considerable interest is the levels of Annexin I, II and IV in a revertant cell line (H69/PR) derived from H69/AR cells that are less resistant to doxorubicin (figure 5). cDNA transfections (transient and stable) of Annexins II-XI, as described below will be carried out to verify the drug resistance of Annexin II-XI-transfected cells.

cDNA transfection and drug sensitivity analyses

In an attempt to investigate the role of P-40 or Annexin I overexpression in drug resistance, the full length gene encoding P-40 or Annexin I was cloned into a mammalian expression vector, pCDNA3 and transfected into MCF-7 cells. MCF-7 cells transfected transiently with pCDNA3 alone or with P-40 gene are cultured for 3 days prior to analysis. The results in figure 6a show a Western blotting of total cell proteins from MCF-7 cells transfected with pCDNA3 vector only or pCDNA3 vector plus P-40 or Annexin I gene (lanes 2 and 3). The results in lane 3 of figure 6a show a 40 kDa protein in MCF-7 cells transfected with pCDNA3 vector plus P-40 or Annexin I. To determine more quantitatively, the level of P-40 transfection following a three day culturing, MCF-7 cells transfected with pCDNA3 plus P-40 or Annexin I full length cDNA were analyzed by indirect immunofluorescence with IPM96 mAb. The results in figure 6b showed the relative number (<5%) of MCF-7 cells that overexpress P-40 or Annexin I. The efficiency of the transient transfection to ~5% of the cells was confirmed following transfection with pCDNA3 containing a beta-gal gene (data not shown).

Having established the expression of P-40 or Annexin I in MCF-7 cells, it was of interest to examine the effect of P-40 or Annexin I overexpression (albeit <5%) on the sensitivity of MCF-7 cells to anticancer drugs. The results in figure 6b and 6c show MCF-7 cells transfected with vector only and with vector plus P-40 incubated with increasing concentrations of Taxol or adriamycin, respectively. Surprisingly, overexpression of P-40 or Annexin I in

MCF-7 cells decreases their sensitivity to Taxol and adriamycin. The analysis as to whether higher levels of P-40 or Annexin I expression will lead to a larger decrease in the sensitivity of transfectant cells to anticancer drugs has also been formerly tested using for stable cell lines expressing P-40-cDNA (see below).

The transient transfected cells were incubated with a chelator (EGTA) or a calcium channel blocker (Verapamil). P-40-protein was shown to be released by the membrane and the EGTA or Verapamil-treated cells were also shown to have reduced drug resistance to taxol or adriamycin. These results suggest that small molecules find utility in the context of the present invention.

DISCUSSION

In this study we have used the monoclonal antibody IPM96, previously shown to detect a 40 kDa protein in MDR cells to screen a λgt11 expression library. Two positive λgt11 clones were identified and their cDNA insert was isolated by PCR and cloned into a TA PCRII vector. Analysis of the nucleotide and amino acid sequence of the 1.4 kb cDNA insert revealed an open reading frame of 346 amino acids that is identical to Annexin I, a known substrate of epidermal growth factor receptor (Wallner et al., 1986, Nature 320: 77-81). *In vitro* expression of IMP96 positive cDNA clone using a transcription-translation retic, lysate followed by immuno precipitation and Western blot analyses of the expressed 40 kDa protein confirmed the identify of the 1.4 kb fragment as P-40. In addition, Northern blot analysis using total RNA from drug sensitive and resistant cells confirmed the overexpression of Annexin I or P-40 mRNA in MDR cells relative to their parental drug sensitive cells.

Besides the similarities in the P-40 and Annexin I molecule masses on SDS-PAGE and the cross-reaction of IPM96 with Annexin I expressed *in vitro*, the identity of the P-40 as Annexin I is consistent with our earlier observations where P-40 was shown to be found both in the membrane

and soluble fractions (Wang et al., 1997, *supra)*. Furthermore, extraction of membrane associated P-40 was resistant to high salt and EDTA and suggests the possibility that some of P-40 may be an integral membrane protein. Interestingly, a similar conclusion was independently suggested for Annexin I in an earlier study . The latter possibility is likely given that annexins I, V, VI and VII possess ion channel activity (Pollard et al., 1988, Proc. Natl. Acad. Sci. USA 85: 2974-2978; Rojas et al., 1990, J. Biol. Chem. 265(24239-24245)). Also consistent with our assignment of P-40 as Annexin I, is the fact that the 35 kDa proteolytic product which has been previously demonstrated to represent the head domain of Annexin I, is highly sensitive to proteolysis (Wang et al., 1994, *supra*).

We have previously shown that P-40 (or annexin I) is highly expressed in several MDR cell lines relative to their parental drug sensitive cells (Wang et al., 1997, *supra*). The MDR cell lines used in our study were previously shown to contain amplified copies of Pgp MDR-1 or MRP genes. The genes encoding Pgp, MRP and P-40 (or annexin I) are localized on chromosome 7, 16 and 9 respectively (Cole et al., 1993, *supra*; Trent and Witkowski, 1987, *supra*; Wallner et al., 1986, *supra*). Therefore, the observed increase in P-40 protein is not due to a co-amplification of P-gp or MRP. In addition, our Slot blot results did not reveal the amplification of P-40 (or annexin I) gene in any of the MDR cell lines where P-gp or MRP are amplified. Furthermore, P-40 (or annexin I) was detected in MDR cell lines that lacked detectable P-gp or MRP. The northern blot analysis of total RNA from drug sensitive and resistant cells revealed an increase in P-40 (or annexin I) mRNA levels in drug resistant cells. Thus, the observed increase in P-40 (or annexin I) protein level in resistant cells is transcriptionally regulated. Alternatively, the increased transcription or mRNA stabilization may govern the overexpression of P-40 in MDR cells. Of interest was the detectable increase in P-40 (or annexin I) mRNA in SKOV3 drug sensitive cells versus that in other drug sensitive cells. However, P-40 (or annexin I) mRNA in SKOV3 cells was four

fold less than that in its drug resistant counterpart (SKOV/VLB$^{1.0}$). In particular, the northern blot results are consistent with the Western blot data, especially those relating to the protein and mRNA levels of P-40 or annexin I in SKOV3 compared with (SKOV/VLB$^{1.0}$).

The observed decrease in P-40 (or annexin I) expression in a revertant cell line (H69/PR) derived from H69/AR (Cole et al., 1992) together with the increase in P-40 in (SKOV/VLB$^{1.0}$) following *in vitro* selection from SKOV3 show a strong correlation between the overexpression of P-40 (or annexin I) and MDR.

Annexin I is a member of a large family of calcium dependent membrane binding proteins that are sometimes referred to as lipocortin, calpectins, endonexins (for review see Raynal et al., 1994, Bioch. Biophys. Acta. 1197: 63-93). Annexins share a similar core domain with four or eight conserved 70 amino acid repeats and an amino terminal domain that varies in length and sequence between the different members of the annexin family Although the physiological function(s) of annexins is not clear, they have been implicated in calcium-regulated exocytosis (Drust et al., 1988, Nature 331: 88-91; Creutz et al., 1987, J. Biol. Chem. 262: 1860-1865). Annexin I has also been shown to mediate the calcium-dependent fusion of liposomes with isolated neutrophil plasma membranes (Meers et al., 1986, Nature 321: 81-84).

In intact cells, annexins are generally phosphorylated in response to varieties of stimuli. Annexin I is phosphorylated by EGF receptor-kinase at tyrosine residues found in the N-terminal head domain (Pepinsky et al., 1986, Nature 321: 81-84) and by protein kinases C and A (Varticovski et al., 1988, Biochem. 27: 3682-3690]. Interestingly, phosphorylation of Annexin I at the amino terminal domain by protein kinase C inhibits its ability to aggregate chromaffin granules (Wang et al., 1986, J. Biol. Chem. 261: 6548-6553.). In adrenal chromaffin cells, Annexin I was shown to be rapidly phosphorylated upon stimulation of cells to secrete normal cell metabolites Wang et al. 1992, Biochem, 31: 9934-9939)

Taken together, our finding that Annexin I is not phosphorylated in MDR cells is consistent with its increased capacity to cause aggregation of membrane vesicles.

In this report we show, for the first time, a direct role of Annexin I overexpression in tumor cells resistance to anticancer drugs. Using transient transfections of MCF-7 tumor cells, we showed that transfection of P-40 or Annexin I cDNA confers resistance to Taxol and adriamycin. Although the levels of resistance towards the latter drugs are only 1.2 to 2.0-fold more than control cells (transfected with vector alone), the results are consistent and are not surprising in light of the percentage (<5%) of transfected cells (figure 6a and data not shown). Furthermore, similar transfection studies of P-gp or MRP cDNA have also shown much lower levels of drug resistant in transfectant cells when compared to selected MDR cells that overexpress similar amounts of these proteins (Zaman et al., 1994, Proc. Nat. Acad. Sc. USA 91(19): 8822-8826; Cole et al., 1994, Cancer Res. 54(22): 5902-5910; Gros et al., 1986, Nature 323: 728-731).

In this study, it is shown that the level of drug resistance of P-40 (or annexin I) transfectants is 2 to 3 fold higher than the transfected with vector alone. It is thus likely that P-40 (or annexin I) confers lower level of drug resistance than P-gp or MRP (3 to 8 fold). However, unlike the high levels of drug resistance seen in *in vitro* drug selected MDR tumour cell lines, low level of drug resistance conferred by different cellular changes (such as P-40) are likely to be clinically relevant. Given the fact that P-40 is expressed at lower level in SKOV3 cells, which are clinically resistant to adriamycin and cisplatin, than that of SKOV/VLB$^{1.0}$ (1000 fold resistance to vinblastine), it is submitted that the observed overexpression of P-40 (or annexin I) is probably an early event in the development of clinical drug resistance. Work is in progress in the investigation of P-40 in clinical tumour samples.

In this study, it is shown, for the first time, that P-40 (or annexin I) confers resistance to anticancer drugs. The results from the

cytotoxicity assays indicate that the drug resistance profile of P-40 (or annexin I) transfectants is similar, but not identical to P-gp or MRP transfectant cells. This apparent difference in the resistance spectrum may be important to the mechanism of P-40 (or annexin I) mediated drug resistance. The mechanism by which P-40 (annexin I) confers drug resistance to anticancer drugs is presently unknown. However, the fact that P-40 cannot be labelled by photoactive analogues of cytotoxic agents (data not shown) and that its amino acid sequence does not encode an ATP binding domain suggest that P-40 (or annexin I) itself does not transport drugs directly to the extracellular environment. The intracellular distribution of P-40 in both *in vitro* selected P-40 expressing cells and in the transfectant cells suggests that the membrane localization of P-40 (annexin I) is essential for its function in drug resistance. Translocation of P-40 from the plasma membrane to cytoplasm after treatment with verapamil and EDTA has been observed in both P-40 expressing cell lines and P-40 cDNA transfectants (data not shown). This finding suggests that P-40 may participate in sequestering drugs from their targets. Alternatively, the intracellular action concentration (eg. Ca^{2+}) may be important for the functions of P-40 (annexin I) in drug resistance. Work is in progress to determine if P-40 induced drug resistance can be reversed by MDR modulators. Given the role of annexins in promoting aggregation of membrane vesicles through Ca^{2+} dependent phospholipid binding, it is proposed that P-40 (annexin I) confers a drug resistance phenotype by aggregation of drug filled membrane vesicles or exocytosis of such drug filed vesicles (Chauffert et al., Cancer Res. 46: 825-830). The observed increase in membrane voculisation in many MDR cell lines (Beck, 1987, Biochem. Phamacol. 36(18): 2879-2887; Sehested et al., 1987 British J. of Cancer 56: 747-751) supports this proposition. Furthermore, P-glycoprotein an MRP have been detected in the endosomal membranes in *in vitro* selected cell lines (Abbaszadegan et al., 1997, Cancer Res. 56(23): 5435-5442; Klohs et al., 1988, Mol. Pharmacol. 34: 180-185). Thus, P-40 (or annexin I) or other members of the annexin family could function together with P-

glycoprotein or MRP to cause the aggregation and possibly exocytosis of drug filled vesicles. Whatever, the mechanism of action of annexins and more particularity of annexin I, the present invention strongly suggests their importance in the establishment of the MDR phenotype in cancer cells. The present invention presents means to further dissect the structure/function relationship of annexins in MDR and provides methods and assays to identify modulators of this MDR phenotype.

The present invention is described in further detail in the following non-limiting examples.

EXAMPLE 1

Characterization of P-40 (or annexin I) MCF-7 stable transfectant cells

To determine if P-40 (or annexin I) alone confers resistance to anticancer drugs, a full length cDNA clone of P-40 (or annexin I) was cloned into pCIN4 eukaryotic expression vector (Figure 7) and transfected into MCF-7 drug sensitive cells. Stable transfectants of P-40 (or annexin I) MCF-7 cells (P-40-MCF-7) were selected in the presence of lethal concentration of G418. Figure 8a shows a Western blot analysis of lysates from P-40-MCF-7 cells probed with the IPM96 monoclonal antibody. The results of the latter western blot shows that P-40 MCF-7 cells express 1/3 less P-40 than the MDR *in vitro* selected MCF-7/Adr cells. However, the distribution pattern of P-40 (or annexin I) in P-40-MCF-7 transfectants is similar to that in MCF-7/Adr cells (Figure 8b). As expected, no detectable levels of P-40 was observed in cells transfected with vector alone.

EXAMPLE 2

Cross resistance patterns of P-40 MCF-7 stable transfectants

Having established the expression of P-40 (or annexin I) in P-40-MCF-7 cells, it was of interest to know the effect of P-40 (or annexin I) on the sensitivity of MCF-7 cells to anticancer drugs. Figure 9 shows the results of

the chemosensitivity assays using P-40-MCF-7 cells in the presence of increasing concentration of anticancer drugs to that of MCF-7 cells transfected with vector alone. Surprisingly, P-40-MCF-7 cells displayed low level of resistance to adriamycin, actinomycin D, Taxol and cisplatin (Figure 9) relative to the cells transfected with vector alone. However, P-40-MCF-7 cells did not show cross-resistance to colchicine and vincristine (data not shown). These results suggest that the overexpression of P-40 (or annexin I) does confer a low level of drug resistance to a variety of anticancer drugs.

In conclusion, the present invention provides convincing evidence that annexin and particularly annexin I are important in the development of the MDR phenotype in cancer cells. The herein presented findings are also important for providing a further understanding of the functions of the annexin family. Analysis of clinical tumour samples for P-40 (or annexin I) expression (primary or relapsed after chemotherapy) will provide further evidence for the diagnostic role of P-40 in clinical drug resistance.

The present description refers to a number of documents, the content of which is herein incorporated by reference.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defind in the appended claims.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule for assessing or modulating multidrug resistance (MDR) in a cell, said nucleic acid molecule encoding an Annexin family member.

2. The isolated nucleic acid molecule of claim 1, wherein said cell is selected from an animal cell, mammalian cell, a human cell, a parasitic cell and a fungal cell.

3. The isolated nucleic acid molecule of claim 1, comprising a nucleic acid sequence which is at least 90% identical to a sequence selected from the group consisting of a nucleotide sequence encoding an Annexin polypeptide selected from Annexin I to Annexin XI, or a nucleotide sequence complementary thereto.

4. The isolated nucleic acid molecule of claim 1, 2 or 3, wherein said Annexin is Annexin I and said Annexin polypeptide is the Annexin I polypeptide which comprises the complete amino acid sequence set forth in SEQ ID NO:2.

5. The isolated nucleic acid molecule of claim 1 or 2, wherein said Annexin is Annexin I and said nucleotide sequence comprises the nucleotide sequence as set forth in SEQ ID NO:1.

6. A method of detecting and/or assessing an Annexin-based MDR phenotype in a sample comprising:

a) contacting said sample with an isolated nucleic acid molecule consisting of 10 to 50 nucleotides specifically hybridizing to RNA and/or DNA encoding an Annexin, wherein said nucleic acid molecule is or is complementary to a nucleotide sequence consisting of at least 10 consecutive nucleotides from the nucleic acid sequence of one of Annexin I to XI, under hybridization conditions; and

b) detecting the presence of said molecule bound to Annexin nucleic acid,

wherein said presence of said Annexin nucleic acid correlates with an Annexin-based MDR in said sample.

7. The method of claim 6, wherein said Annexin-based MDR is an Annexin I-based MDR.

8. A kit for detecting and/or quantifying an Annexin-based MDR phenotype in a sample, comprising at least one container means having disposed therein an isolated nucleic acid molecule consisting of 10 to 50 nucleotides specifically hybridizing to RNA and/or DNA encoding an Annexin, wherein said nucleic acid molecule is or is complementary to a nucleotide sequence consisting of at least 10 consecutive nucleotides from the nucleic acid sequence of one of Annexin I to XI; and wherein said presence and/or said quantification of Annexin nucleic acid sequence correlates with an Annexin-based MDR in said sample.

9. The kit of claim 8, wherein said nucleotides specifically hybridize to Annexin I, thereby detecting and/or quantifying an Annexin I-based MDR.

10. A recombinant vector for modulating and/or increasing Annexin-based MDR in a cell comprising the isolated nucleic acid molecule of claim 1 operably linked to a promoter element.

11. The recombinant vector of claim 10, wherein Annexin is Annexin I.

12. The recombinant vector of claim 11, being pCDNA3/P-40 or pC1N4P-40.

13. A cell that contains the recombinant vector of claim 10, 11 or 12; said cell having been rendered multidrug resistant (MDR) by said expression of said Annexin nucleic acid molecule.

14. The cell of claim 13, having been rendered MDR by the expression of recombinant Annexin I.

15. A method of identifying compounds that affect Annexin-based MDR in a cell, said method comprising:

a) incubating said cell in the presence of a potential Annexin-based MDR-affecting compound in the presence and absence of a drug; and

b) assessing the effect of said compound on the resistance of said cell to said drug.

16. The method of claim 14, wherein said cell is a cell in accordance with one of claims 13 or 14.

17. The method of claim 15 or 16, wherein said compound is selected from the group consisting of a nucleic acid molecule encoding an Annexin variant, or a part thereof, a dominant negative mutant of an Annexin, a mutant Annexin, an antibody to Annexin, a peptide, and a small molecule.

18. The method of claim 17, wherein said compound is an Annexin I antisense molecule.

19. The method of one of claims 15, 16, 17 or 18, wherein said drug is an anticancer drug.

20. A method of reducing Annexin-based MDR in a cell comprising: administering thereto a therapeutically effective amount of a compound selected from the group consisting of a nucleic acid molecule, a dominant negative mutant of an Annexin, a mutant Annexin protein, an antibody to Annexin, a peptide, and a small molecule.

21. The method of claim 20, wherein said Annexin-based MDR is Annexin I-based.

22. The method of claim 21, wherein said compound is an Annexin I antisense molecule.

23. The method of claim 21, wherein said compound is a calcium chelator or a calcium channel blocker.

24. A pharmaceutical composition for reducing MDR in a cell comprising an Annexin-based MDR affecting compound together with a pharmaceutically acceptable carrier.

25. The pharmaceutical composition of claim 24, wherein said Annexin-based MDR is Annexin I-based, and said compound is an Annexin I-based MDR affecting compound.

26. A method of diagnosing the presence or predisposition of Annexin-based MDR in a patient comprising:

a) taking a sample from said patient;

b) determining the amount of Annexin protein and/or nucleic acid in said sample;

c) diagnosing the presence or predisposition of Annexin-based MDR in said patient, wherein an increased amount of said Annexin protein and/or nucleic acid in said sample as compared to a control sample indicates the presence or predisposition towards Annexin-based MDR.

27. The method of claim 26, wherein said Annexin-based MDR is Annexin I-based and said determining is a determination of Annexin I protein and/or nucleic acid.

28. A method of diagnosing the presence or predisposition of Annexin-based MDR in a pathogen comprising:

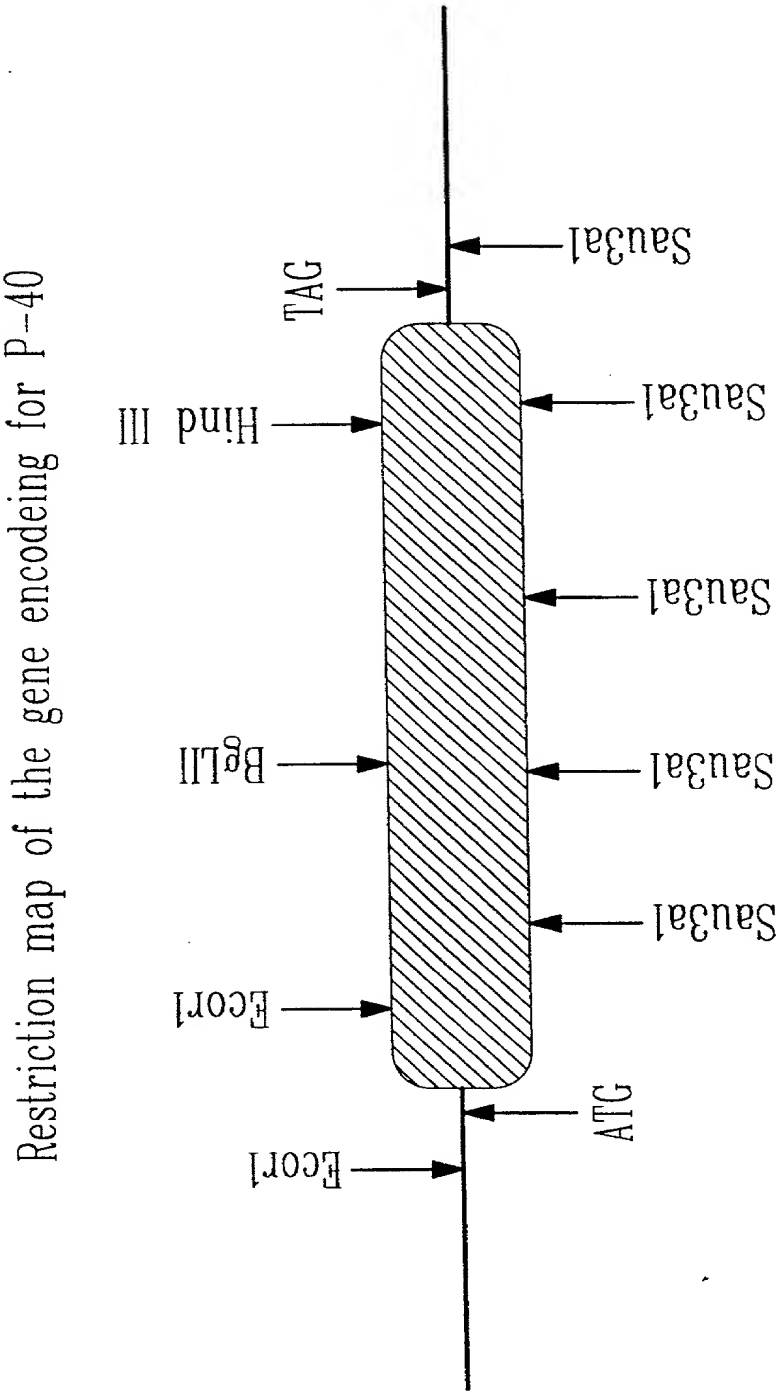
a) taking a sample from said pathogen;

b) determining the amount of Annexin protein and/or nucleic acid in said sample;

c) diagnosing the presence or predisposition of Annexin-based MDR in said pathogen, wherein an increased amount of said Annexin protein and/or nucleic acid in said sample as compared to a control sample indicates the presence or predisposition towards Annexin-based MDR.

29. The method of claim 28, wherein said Annexin-based MDR is Annexin I-based and said determining is a determination of Annexin I protein and/or nucleic acid.

FIG - 1A


Restriction map of the gene encodeing for P-40
Sau3al
TAG
Hind III
Sau3al
Sau3al
BgLII
Sau3al
Sau3al
EcorI
ATG
EcorI

```
ORIGIN
      1 agtgtgaaat cttcagagaa gaatttctct ttagttcttt gcaagaaggt agagataaag
     61 acacttttcc aaaaatggca atggtatcag aattcctcaa gcaggcctgg tttattgaaa
    121 atgaagagca ggaatatgtt caaactgtga agtcatccaa aggtggtccc ggatcagcgg
    181 tgagcccccta tcctacctc aatccatcct cggatgtcgc tgccttgcat aaggccataa
    241 tggttaaagg tgtggatgaa gcaaccatca ttgacattct aactaagcga aacaatgcac
    301 agcgtcaaca gatcaaagca gcatatctcc aggaaacagg aaagcccctg gatgaaacac
    361 ttaagaaagc ccttacaggt cacattgagg aggttgtttt agctctgcta aaaactccag
    421 cgcaatttga tgctgatgaa cttcgtgctg ccatgaaggg ccttggaact gatgaagata
    481 ctctaatttga gattttggca tcaagaacta acaaagaaat cagagacatt aacagggtct
    541 acagagagga actgaagaga gatctggcca aagacataac ctcagacaca tctgggagatt
    601 ttcggaacgc tttgctttct cttgctaagg gtgaccgatc tgagggactt ggtgtgaatg
    661 aagacttggc tgattcagat gccagggcct tgtatgaagc aggagaaagg agaaagggga
```

Fig - 1B

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 721 cagacgtaaa cgtgttcaat accatccttа ccaccagaag ctatccacaa cttcgcagag
 781 tgtttcagaa atacaccaag tacagtaagc atgacatgaa caaagttctg gacctggagt
 841 tgaaaggtga cattgagaaa tgcctcacag ctatcgtgaa gtgcgccaca agcaaaccag
 901 ctttctttgc agagaagctt catcaagcca tgaaagggtgt tggaactcgc cataaggcat
 961 tgatcaggat tatggtttcc cgttctgaaa ttgacatgaa tgatatcaaa gcattctatc
1021 agaagatgta tggtatctcc cttgccaaag ccatcctgga tgaaaccaaa ggagattatg
1081 agaaaatcct ggtggctctt tgtggaggaa actaaacatt cccttgatgg tctcaagcta
1141 tgatcagaag actttaatta tatatttca tcctataagc ttaaatagga aagtttcttc
1201 aacaggatta cagtgtagct acctacatgc tgaaaaatat agccctttaa tcatttttat
1261 attataactc tgtataatag agataagtcc attttttaaa aatgtttttcc ccaaaccata
1321 aaaccctata caagttgttc tagtaacaat acatgagaaa gatgtctatg tagctgaaaa
1381 taaaatgacg tcacaagac
```

FIG - 1C

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MAMVSEFLKQAWFIENEEQEYVQTVKSSKGGPGSAVSPYPTFNPSSDVAALHKAIMVK

GVDEATIIDILTKRNNAQRQQIKAAYLQETGKPLDETLKKALTGHLEEVVLALLKTPA

QFDADELRAAMKGLGTDEDTLIEILASRTNKEIRDINRVYREELKRDLAKDITSDTSG

DFRNALLSLAKGDRSEDFGVNEDLADSDARALYEAGERRKGTDVNVFNTILTTRSYPQ

LRRVFQKYTKYSKHDMNKVLDLELKGDIEKCLTAIVKCATSKPAFFAEKLHQAMKGVG

TRHKALIRIMVSRSEIDMNDIKAFYQKMYGISLCQAILDETKGDYEKILVALCGGN

Fig - 10

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                10        20        30        40        50        60
                 *         *         *         *         *         *
3'  GTAGCGACCGGCGCTCAGCTGGAATTCGCGGCCGCGTCGATTTTTTTGTCTTGTGACGTCATTTTATT
5'  CATCGCTGGCCGCGAGTCGACCTTAAGCGCCGGCGCAGCTAAAAAAACAGAACACTGCAGTAAAATAA

      A  T  G  A  Q  L  E  F  A  A  A  S  I  F  L  S  C  D  V  I  L  F>
    *  R  P  A  L  S  W  N  S  R  P  R  R  F  F  C  L  V  T  S  F  Y>
      S  D  R  R  S  A  G  I  R  G  R  V  D  F  F  V  L  *  R  H  F  I>
  <Y  R  G  A  S  L  Q  F  E  R  G  R  R  N  K  Q  R  T  V  D  N  *  K
    <A  V  P  A  *  S  S  N  A  A  A  D  I  K  K  D  Q  S  T  M  K  N
    <L  S  R  R  E  A  P  I  R  P  R  T  S  K  K  T  K  H  R  *  K  I

                90        100       110       120       130
                 *         *         *         *         *
TTCAGCTACATAGACATCTTTCTCATGTTACTAGAACAACTTGTATAGGGTTTTATGGTTTGGGGAAA
AAGTCGATGTATCTGTAGAAAGAGTACAATGATCTTGTTGAACATATCCCAAAATACCAAACCCCTTT
      S  A  T  *  T  S  F  S  C  Y  *  N  N  L  Y  R  V  L  W  F  G  E>
  F  Q  L  H  R  H  L  S  H  V  T  R  T  T  C  I  G  F  Y  G  L  G  K>
    F  S  Y  I  D  I  F  L  M  L  L  E  Q  L  V  *  G  F  M  V  W  G  K>
  <*  S  C  L  C  R  E  *  T  V  L  V  V  Q  I  P  N  *  P  K  P  F
  <E  A  V  Y  V  D  K  E  H  *  *  F  L  K  Y  L  T  K  H  N  P  S  F
  <K  L  *  M  S  M  K  R  M  N  S  S  C  S  T  Y  P  K  I  T  Q  P  F
```

Fig-1E

```
     140        150        160        170        180        190        200
      *          *          *          *          *          *          *
ACATTTTTAAAAAATGGACTTATCTCTATTATACAGAGTTATAATATAAAAATGATTTAAAGGCTATA
TGTAAAAATTTTTTACCTGAATAGAGATAATATGTCTCAATATTATATTTTTACTAAATTTCCGATAT
N  I  F  K  K  W  T  Y  L  Y  Y  T  E  L  *  Y  K  N  D  L  K  A  I>
 T  F  L  K  N  G  L  I  S  I  I  Q  S  Y  N  I  K  M  I  *  R  L  Y>
  H  F  *  K  M  D  L  S  L  L  Y  R  V  I  I  *  K  *  F  K  G  Y>
<V  N  K  F  F  P  S  I  E  I  I  C  L  *  L  I  F  I  I  *  L  S  Y
 <M  K  L  F  H  V  *  R  *  *  V  S  N  Y  Y  L  F  S  K  F  A  I
 <C  K  *  F  I  S  K  D  R  N  Y  L  T  I  I  Y  F  H  N  L  P  *  I

       210        220        230        240        250        260        270
        *          *          *          *          *          *          *
TTTTTCAGCATGTAGGTAGCTACACTGTAATCCTGTTGAAGAAACTTTCCTATTTAAGCTTATAGGAT
AAAAAGTCGTACATCCATCGATGTGACATTAGGACAACTTCTTTGAAAGGATAAATTCGAATATCCTA
 F  F  S  M  *  V  A  T  L  *  S  C  *  R  N  F  P  I  *  A  Y  R  M>
  F. S  A  C  R  *  L  H  C  N  P  V  E  E  T  F  L  F  K  L  I  G>
I  F  Q  H  V  G  S  Y  T  V  I  L  L  K  K  L  S  Y  L  S  L  *  D>
<K  E  A  H  L  Y  S  C  Q  L  G  T  S  S  V  K  R  N  L  S  I  P  H
<N  K  L  M  Y  T  A  V  S  Y  D  Q  Q  L  F  K  G  I  *  A  *  L  I
 <K  *  C  T  P  L  *  V  T  I  R  N  F  F  S  E  *  K  L  K  Y  S
```

FIG. 1F

```
      280       290       300       310       320       330       340
       *         *         *         *         *         *         *
GAAAATATATAATTAAATTAAGTCTTCTGATCATAGCTTGAGACCATCAAGGGAATGTTTAGTTTCCTCCAC
CTTTTATATATTAATTTCAGAAGACTAGTATCGAACTCTGGTAGTTCCCTTACAAATCAAAGGAGGTG
  K  I  Y  Y  N  *  S  L  L  I  I  A  *  D  H  Q  G  G  N  V  *  F  P  P  P>
* K  Y  Y  I  I  K  V  F  *  S  *  L  E  T  I  K  G  M  F  S  F  L  H>
  E  N  I  *  L  K  S  S  D  H  S  L  R  P  S  R  E  C  L  V  S  S  T>
 <F  Y  I  I  L  T  K  Q  D  Y  S  S  V  M  L  P  I  N  L  K  R  W
<F  I  Y  L  *  L  R  R  I  M  A  Q  S  W  *  P  F  T  *  N  G  G  C
<S  F  I  Y  N  F  D  E  S  *  L  K  L  G  D  L  S  H  K  T  E  E  V

      350       360       370       380       390       400
       *         *         *         *         *         *
AAAGAGCCACCAGGATTTTCTCATAATCTCCTTTGGTTTCATCCAGGATGGCTTGGCAAAGGAGATA
TTTCTCGGTGGTCCTAAAAGAGTATTAGAGGAAACCAAAGTAGGTCCTACCGAACCGTTTCCCTCTAT
Q  R  A  T  R  I  F  S  *  S  P  L  V  S  S  R  M  A  W  Q  R  E  I>
K  E  P  P  G  F  S  H  N  L  L  W  F  H  P  G  W  L  G  K  G  R  Y>
 K' S  H  Q  D  F  L  I  I  S  F  G  F  I  Q  D  G  L  A  K  G  D>
<L  S  G  G  P  N  E  *  L  R  R  Q  N  *  G  P  H  S  P  L  P  L  Y
<L A V L I K E Y D G K T E D L I A Q C L S I
<F  L  W  S  K  R  M  I  E  K  P  K  M  W  S  P  K  A  F  P  S  V
```

Fig - 16

```
410         420         430         440         450         460         470
 *           *           *           *           *           *           *
CCATACATCTTCTGATAGAATGCTTTGATATCATTCATGTCAATTTCAGAACGGGAAACCATAATCCT
GGTATGTAGAAGACTATCTTACGAAACTATAGTAAGTACAGTTAAAGTCTTGCCCTTTGGTATTAGGA
 P  Y  I  F  *  *  N  A  L  I  S  F  M  S  I  S  E  R  E  T  I  I  L>
  H  T  S  S  D  R  M  L  *  Y  H  S  C  Q  F  Q  N  G  K  P  *  S>
T  I  H  L  L  I  E  C  F  D  I  I  H  V  N  F  R  T  G  N  H  N  P>
<W  V  D  E  S  L  I  S  Q  Y  *  E  H  *  N  *  F  P  F  G  Y  D  Q
<G  Y  M  K  Q  Y  F  A  K  I  D  N  M  D  I  E  S  R  S  V  M  I  R
  <M  C  R  R  I  S  H  K  S  I  M  *  T  L  K  L  V  P  F  W  L  G

 480         490         500         510         520         530         540
  *           *           *           *           *           *           *
GATCAATGCCTTATTGCGAGTTCCAACACCTTTCATGGCTTGATGAAGCTTCTCTGCAAAGAAAGCT 5'
CTAGTTACGGAATAACGCTCAAGGTTGTGGAAAGTACCGAACTACTTCGAAGAGACGTTTCTTTCGA 3'
  I  N  A  L  L  R  V  P  T  P  F  M  A  *  *  S  F  S  A  K  K  A>
* S  M  P  Y  C  E  F  Q  H  L  S  W  L  D  E  A  S  L  Q  R  K  L>
 D  Q  C  L  I  A  S  S  N  T  F  H  G  L  M  K  L  L  C  K  E  S  X>
 <D  I  G  *  Q  S  N  W  C  R  E  H  S  S  S  A  E  R  C  L  F
<I  L  A  K  N  R  T  G  V  G  K  M  A  Q  H  L  K  E  A  F  F  A
<S  *  H  R  I  A  L  E  L  V  K  *  P  K  I  F  S  R  Q  L  S  L
```

Fig. 1H

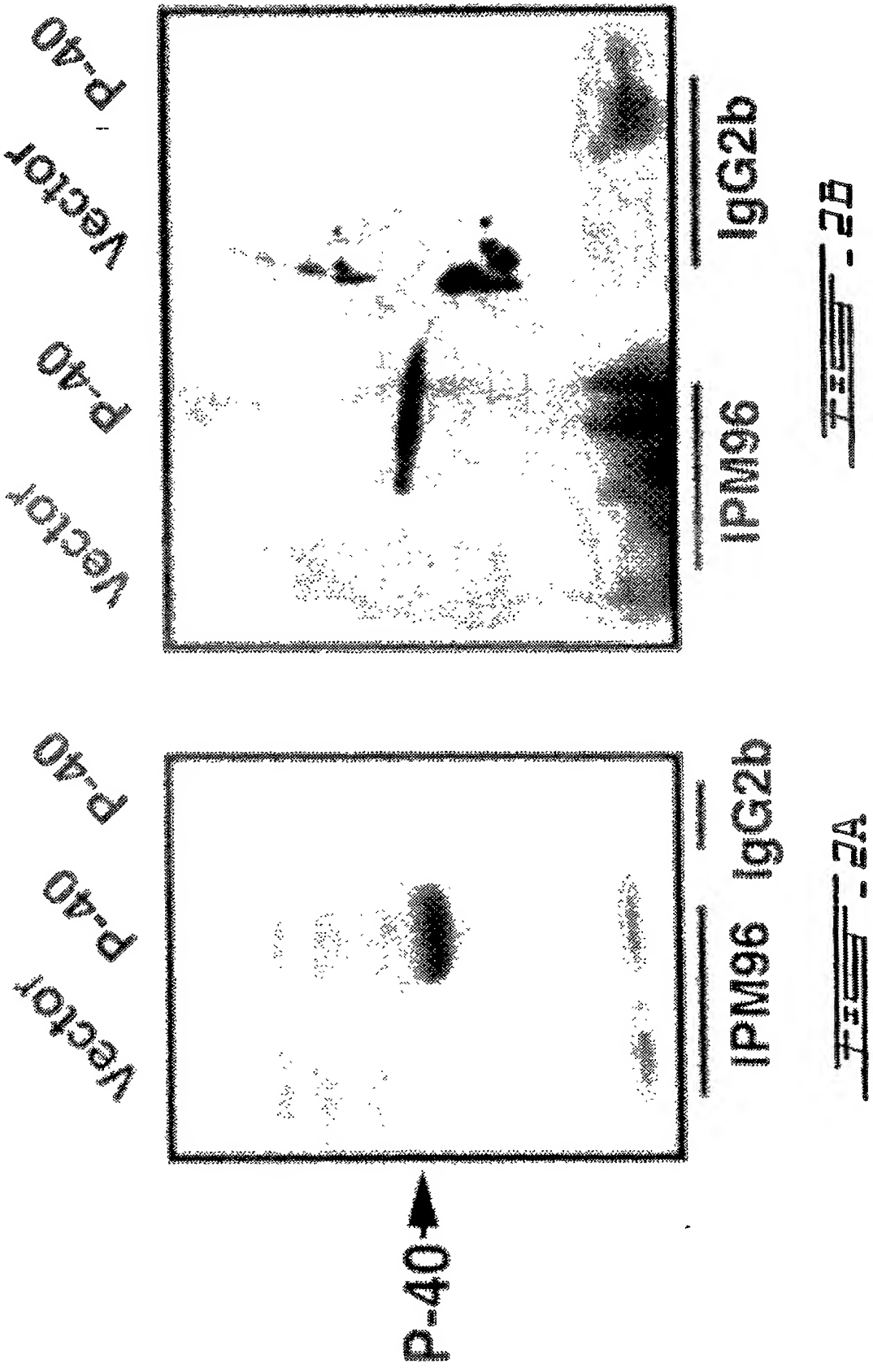
P-40
IPM96
IgG2b
Vector
P-40
Vector
P-40
IPM96
IgG2b
Vector
P-40

FIG - 2A

FIG - 2B

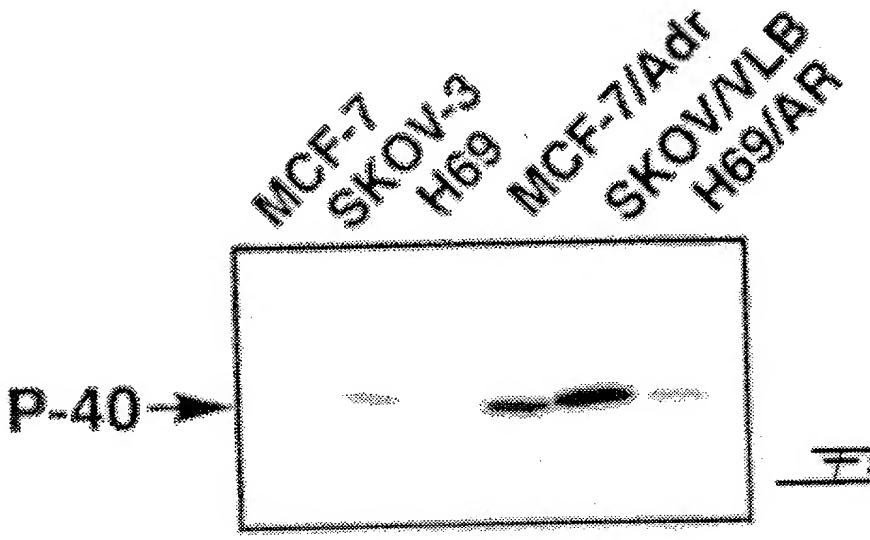
MCF-7
SKOV-3
H69
MCF-7/Adr
SKOV/VLB
H69/AR
P-40

FIG. 3A

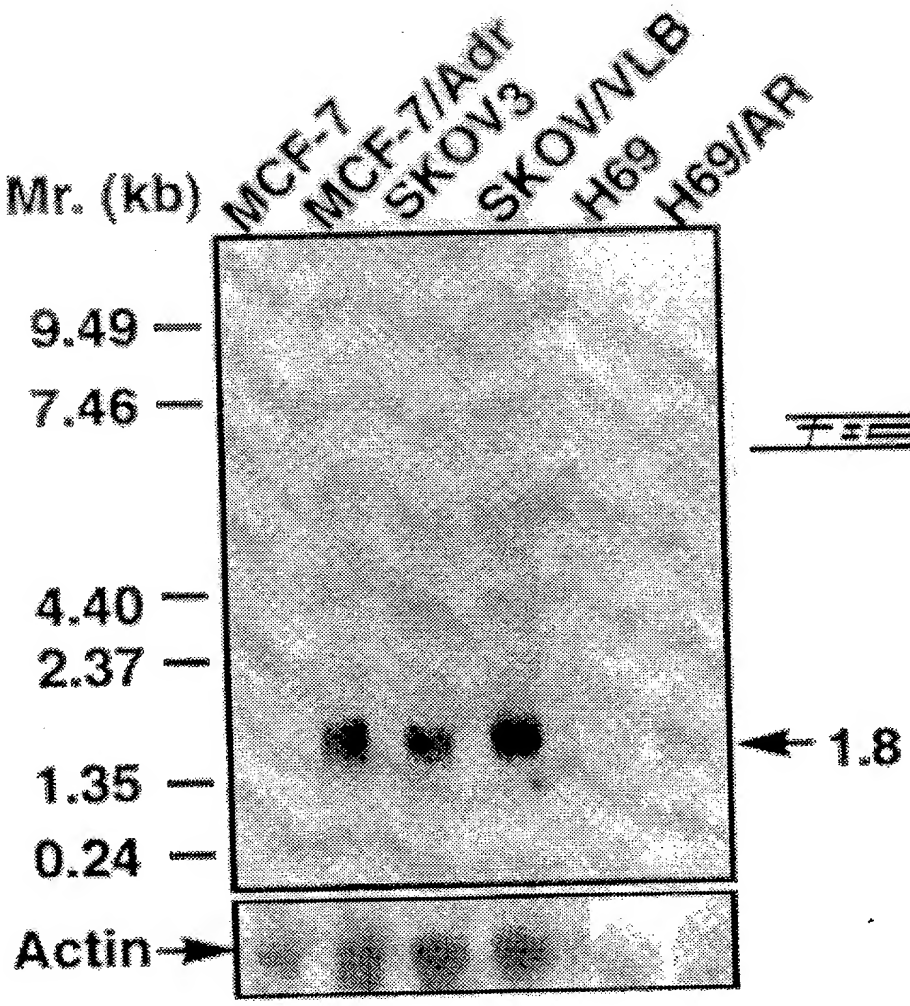
Mr. (kb)
MCF-7
MCF-7/Adr
SKOV3
SKOV/VLB
H69
H69/AR
9.49
7.46
4.40
2.37
1.35
0.24
1.8
Actin

FIG. 3B

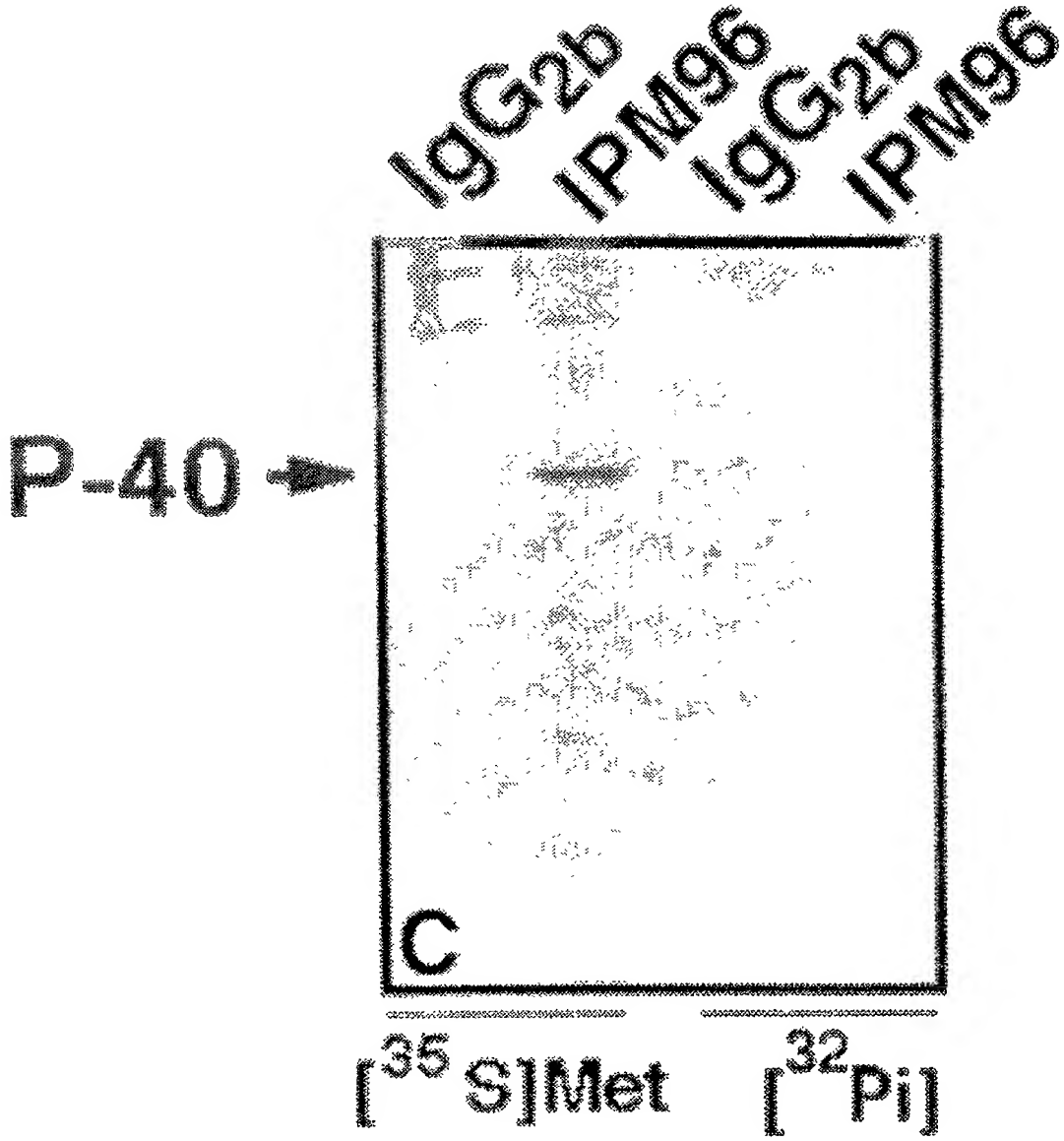

FIG. 4

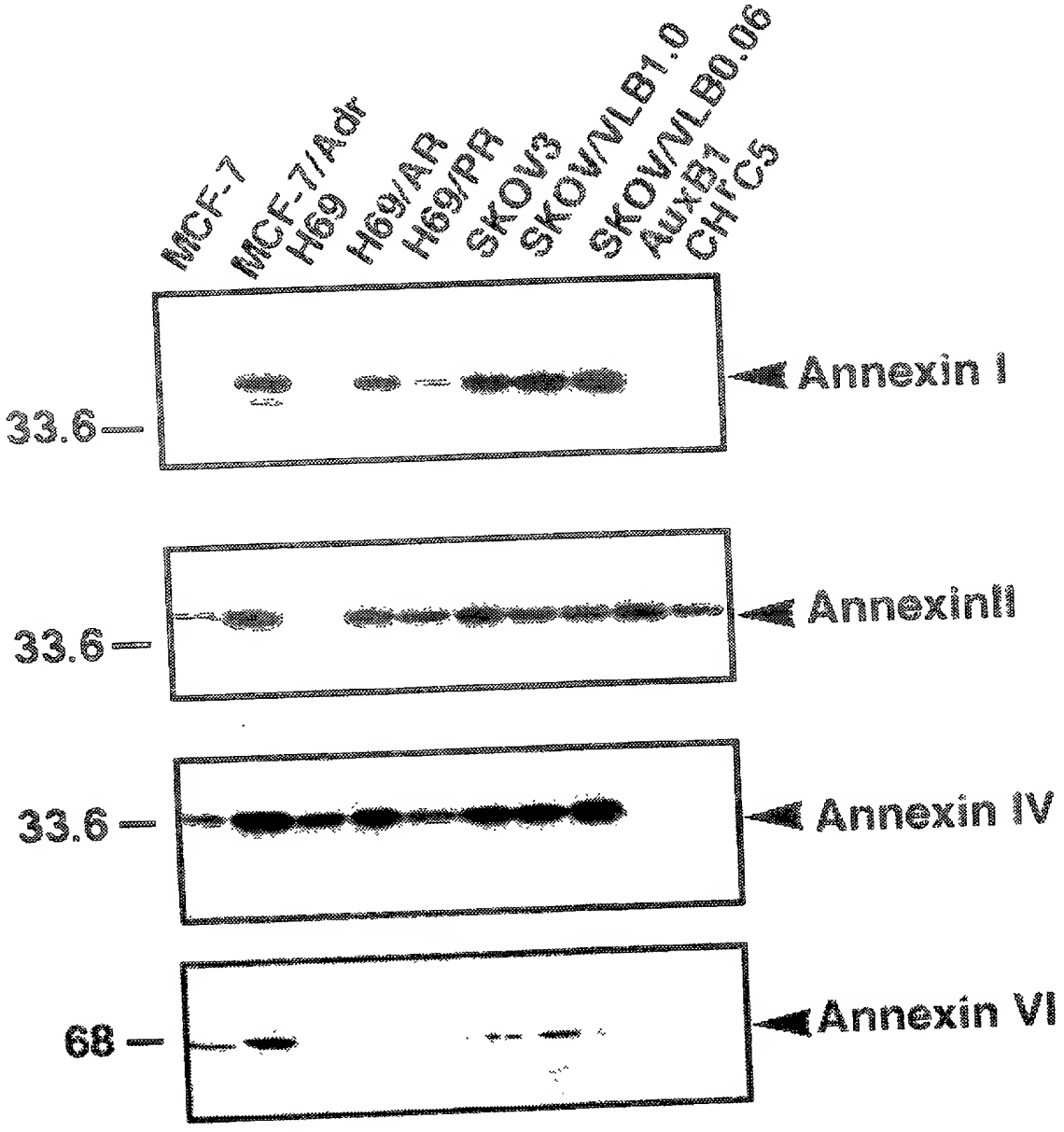
MCF-7
MCF-7/Adr
H69
H69/AR
H69/PR
SKOV3
SKOV/VLB1.0
SKOV/VLB0.06
AuxB1
CHrC5
33.6
Annexin I
33.6
AnnexinII
33.6
Annexin IV
68
Annexin VI

FIG. 5

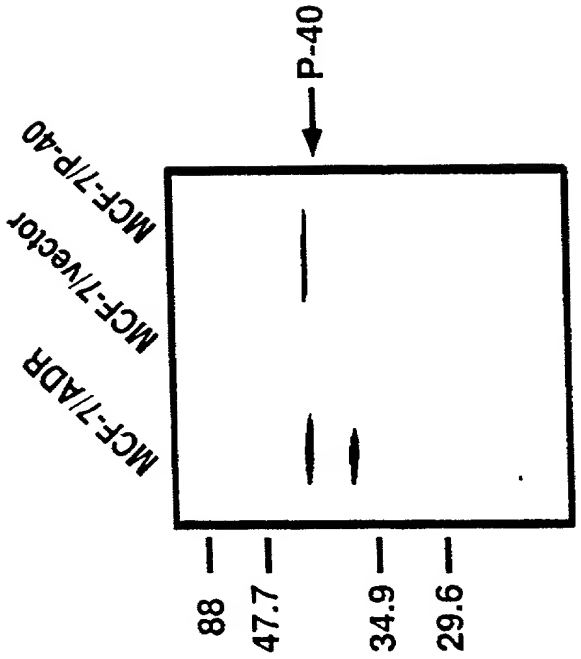

Fig - 6A

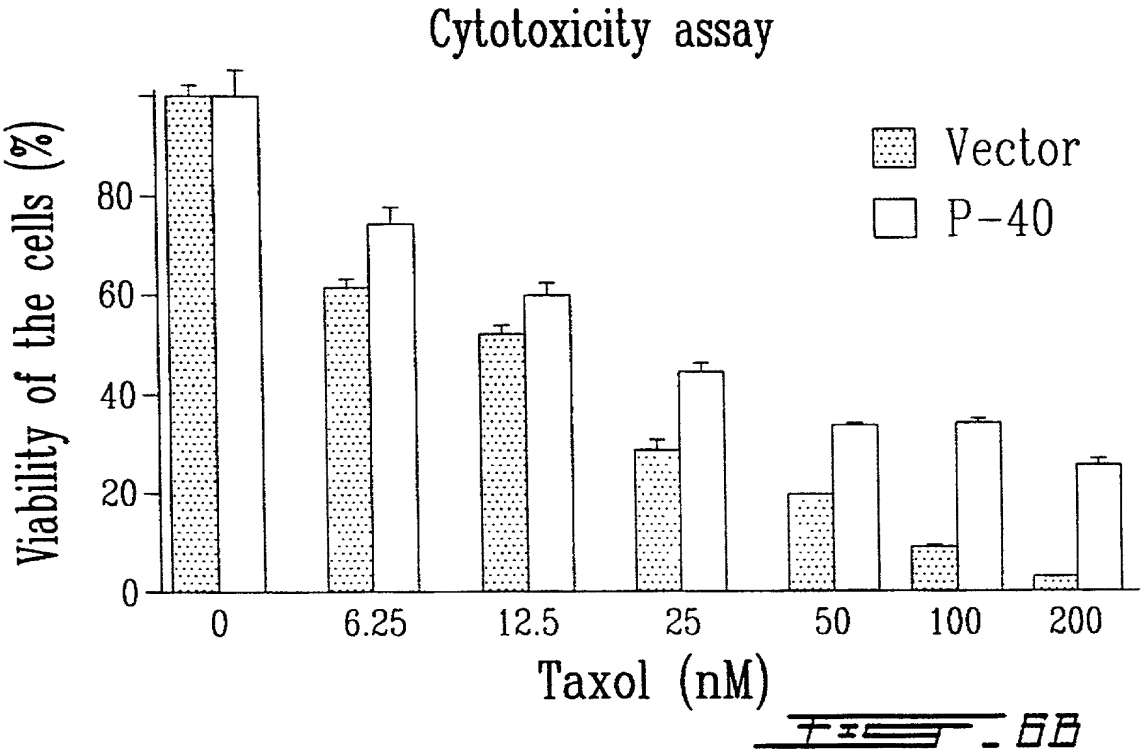
Cytotoxicity assay
Viability of the cells (%)
0
20
40
60
80
Vector
P-40
0
6.25
12.5
25
50
100
200
Taxol (nM)

FIG. 6B

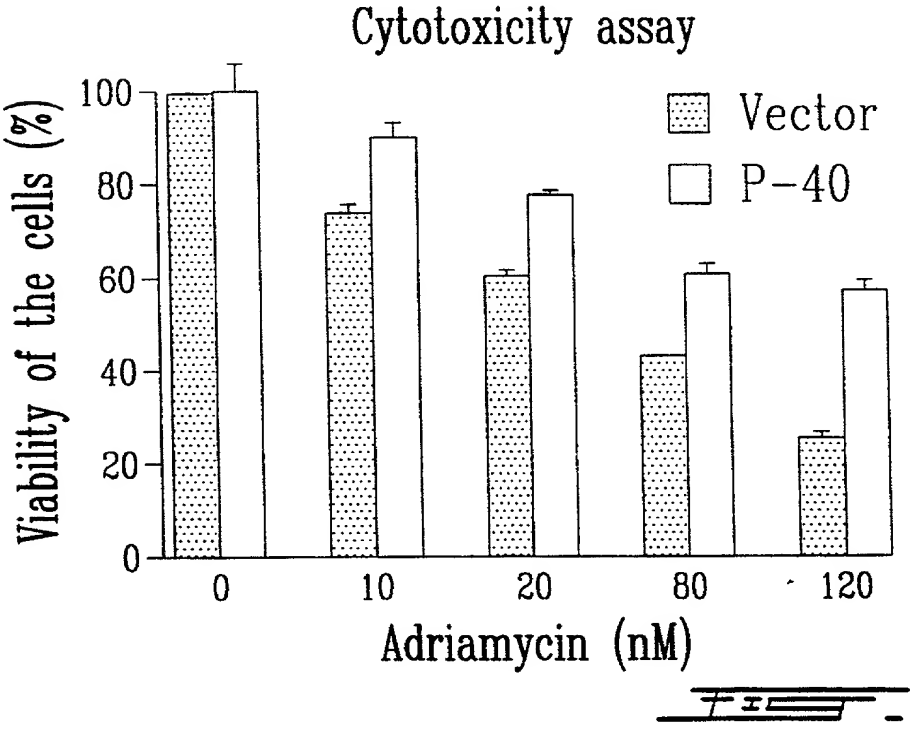
Cytotoxicity assay
Viability of the cells (%)
0
20
40
60
80
100
Vector
P-40
0
10
20
80
120
Adriamycin (nM)

FIG. 6C

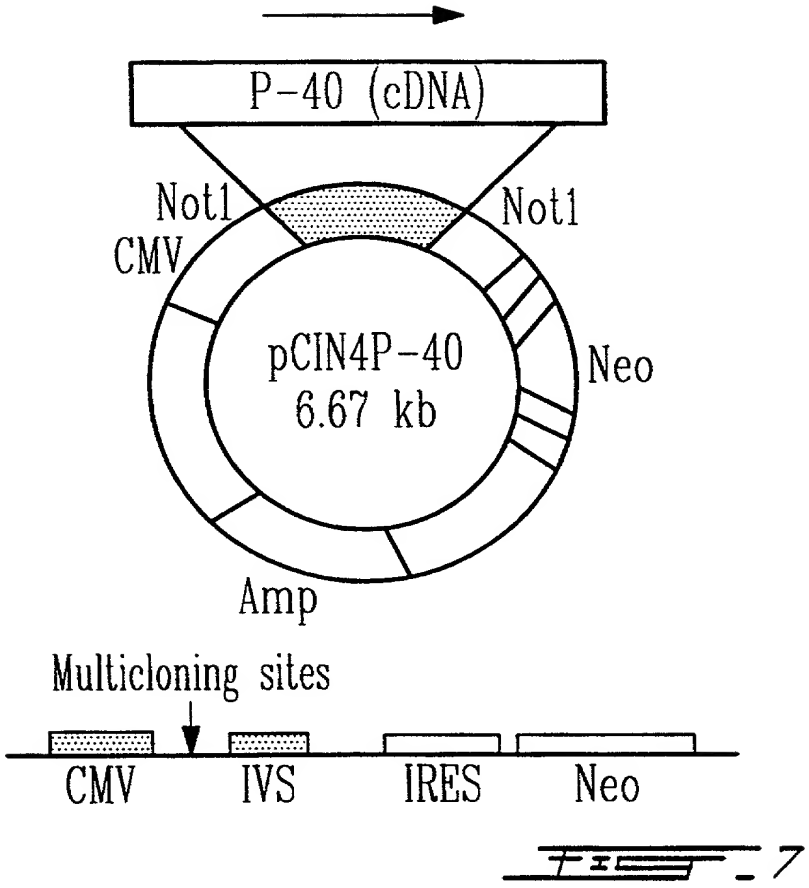
P-40 (cDNA)
Not1
Not1
CMV
pCIN4P-40
6.67 kb
Neo
Amp
Multicloning sites
CMV
IVS
IRES
Neo

Fig. 7

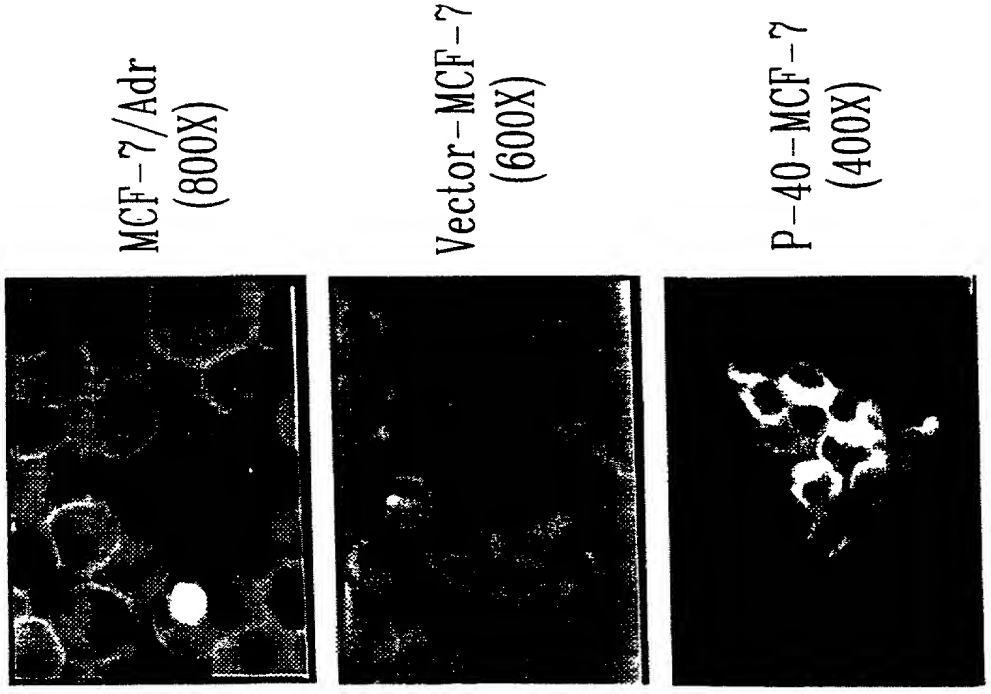
MCF-7/Adr
(800X)
Vector-MCF-7
(600X)
P-40-MCF-7
(400X)

FIG - 8B

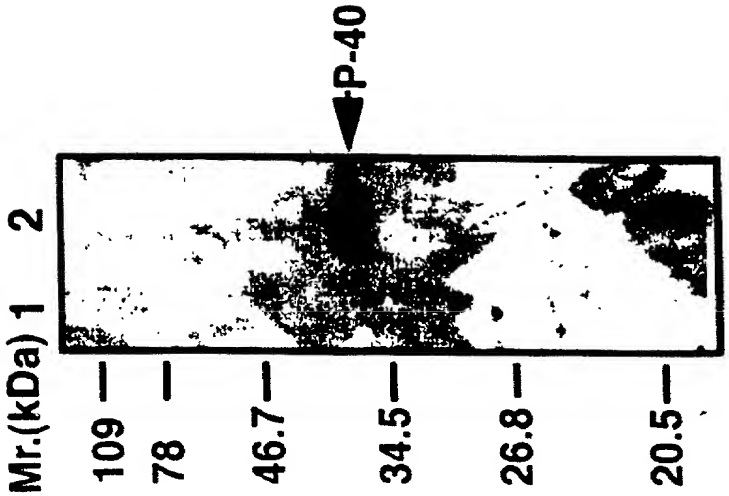
Mr.(kDa) 1 2
P-40
109
78
46.7
34.5
26.8
20.5

FIG - 8A

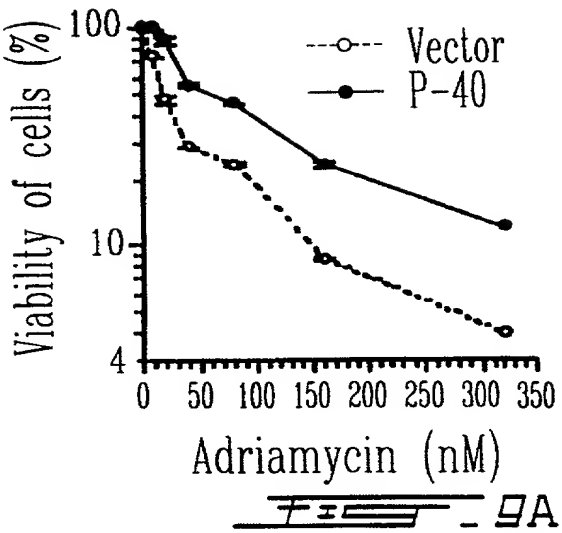

Fig. 9A

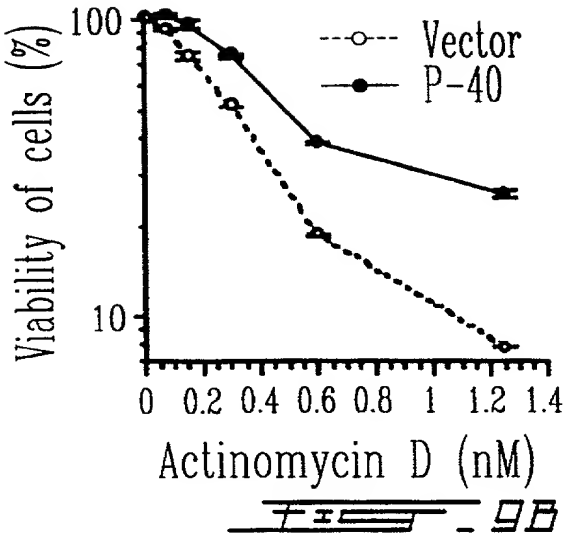

Fig. 9B

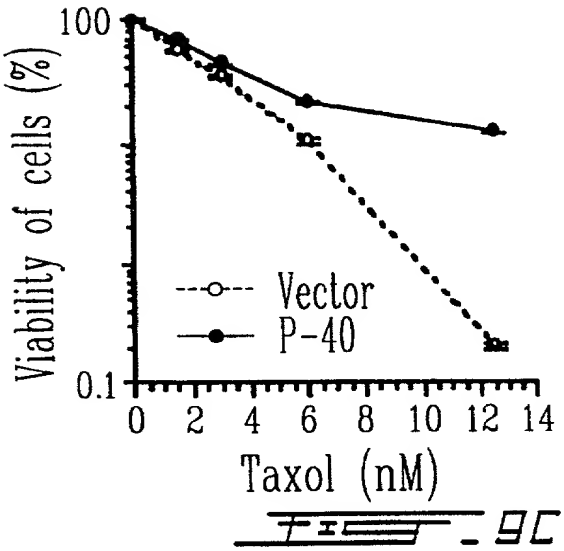

Fig. 9C

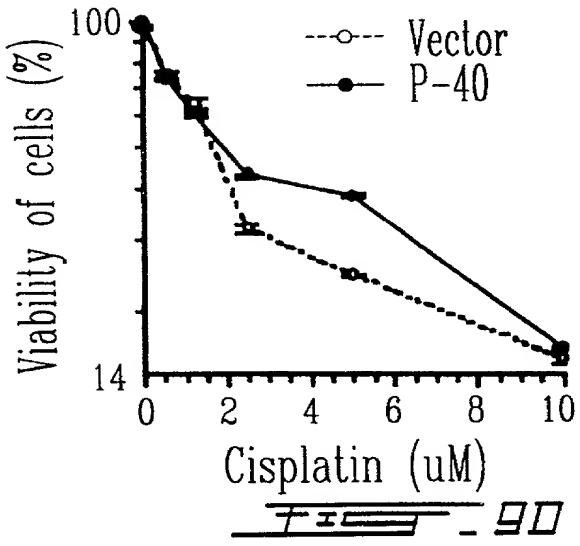

Fig. 9D

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (INCLUDES Reference to PCT International Application)	ATTORNEY DOCKET NUMBER 6x1000 00013

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

P-40/ANNEXIN I AND RELATED PROTEINS AND THEIR ROLE IN MULTIDRUG RESISTANCE

the specification of which (check only one item below):

[] is attached hereto.

[] was filed as U.S. Patent Application Serial Number __
on ___.
as amended on ____ (if applicable).

[X] was filed as a PCT international application number PCT/CA98/00992 on 26 OCT 1998
as amended under PCT Article 19 on __ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications for which priority is claimed:

PRIOR FOREIGN PATENT APPLICATION(S) AND ANY PRIORITY CLAIMED UNDER 35 U.S.C. §119:

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (Day Month Year)	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/CA98/00992	26 October 1998	[X] YES [] NO
Canada	2 219 299	24 October 1997	[X] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER: 641030.20015

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

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U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NUMBER	PCT FILING DATE	U.S. SERIAL NUMBERS			

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201	FULL NAME OF INVENTOR	FAMILY NAME: GEORGES	FIRST GIVEN NAME: Elie	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: [illegible]	STATE OR COUNTRY: Canada CAX	COUNTRY OF CITIZENSHIP: Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: [illegible]	CITY: [illegible]	STATE & ZIP CODE/COUNTRY: Canada [illegible]
202	FULL NAME OF INVENTOR	FAMILY NAME: WANG	FIRST GIVEN NAME: Ying	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: [illegible]	STATE OR COUNTRY: Canada CAX	COUNTRY OF CITIZENSHIP: Canada
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203	FULL NAME OF INVENTOR	FAMILY NAME:	FIRST GIVEN NAME:	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY:	STATE OR COUNTRY:	COUNTRY OF CITIZENSHIP:
	POST OFFICE ADDRESS	POST OFFICE ADDRESS:	CITY:	STATE & ZIP CODE/COUNTRY:

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SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
Georges [signature]	Ying Wang [signature]	
DATE: June 22, 2000	DATE: June 21, 2000	DATE:

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